WNT SIGNALING IN HYPOTHALAMIC NEURAL PROGENITOR DIFFERENTIATION

by

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ABSTRACT

Postdevelopmental neurogenesis is a general phenomenon found in all vertebrate brains, and is of potential therapeutic interest for the treatment of human degenerative diseases. It is known that the rostral migratory stream (RMS) and the subgranular zone (SGZ) of the dentate gyrus maintain constitutive neurogenesis in the adult mammalian brain, and recent preliminary studies have shown that a lower rate of adult neurogenesis persists in previously uncharacterized regions of the brain including hypothalamus. The key regulators for the differentiation of embryonic/adult neural stem cells have been intensely studied, but their precise roles remain highly controversial.

When I initially began this project, the Wnt signaling pathway is known to play a significant role in developmental neurogenesis, and Wnt activity is evident in several regions of the brain that maintain constitutive neurogenesis. However, the specific role of Wnt signaling in zebrafish hypothalamic neurogenesis was unknown. Therefore, I investigated the precise pattern and function of hypothalamic Wnt activity, and I performed a complete functional analysis in the zebrafish and adult mouse hypothalamus.

My work characterized Wnt expression patterns in the zebrafish and adult mouse hypothalamus, and uncovered two major stages of neurogenic development in the zebrafish hypothalamus: 1) Wnt activity is first required for the proliferation of

unspecified hypothalamic progenitors in the embryo; 2) Wnt activity is required again, transiently, for the differentiation of neural progenitors throughout the life of the differentiation of neural progenitors throughout the life of the animal. In this second stage, our findings suggest that Wnt activity is required for the differentiation of neural progenitors, and is subsequently down-regulated to promote the transition from progenitor to precursor and finally, postmitotic neuron. Additionally, I discovered that Wnt signaling plays a conserved role in the differentiation of Wnt-responsive neural progenitors arising from the parenchymal zone in the adult mouse hypothalamus, and in inhibiting the differentiation of tanycytes arising from ventricular progenitors in both the mice and zebrafish hypothalamus. Thus my studies have established the vertebrate hypothalamus as a model for Wnt-regulated postembryonic neural progenitor differentiation, and have demonstrated that the general existence and function of Wnt signaling in this tissue is evolutionarily conserved across both developmental stages and species.

This thesis is dedicated to my parents in China, 王国豪和陶诵.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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CHAPTER 1

INTRODUCTION

Why we study brain neurogenesis

The human brain is the most sophisticated organ of the vertebrate kingdom. The brain contains approximately 100 billion neurons generated via developmental neurogenesis. However, neurogenesis is not solely a developmental phenomenon: neurogenesis is also observed in the adult brain.[1] Researchers have intensively studied the regulation of neurogenesis at both embryonic and postembryonic stages, with the hopes of curing neurodegenerative diseases via the ability of the brain to undergo repair and modification during life, and to decipher how the function of brain is related to the generation of neurons.

Many neurodegenerative diseases including Parkinson's, Alzheimer's, and Huntington's result from the loss of functional neurons. Currently, there are two modes of treatment under intensive investigation: on one side, researchers are working on preventing abnormal neuronal/neurite death; on the other, researchers are developing cell replacement therapy that includes the injection of neural progenitors or of directed differentiated neurons from an endogenous source. In order to realize the latter idea, a reliable way to control each step of neurogenesis, especially the differentiation of adult neural progenitors, is required. Although there are several sources in multiple adult brain regions from which to acquire neural progenitors, the function of the signaling pathways that induce their normal differentiation have not been clearly illustrated, even after decades of studies.[1]

In general, neurogenesis can be divided into three steps (Fig. 1.1): The first step is

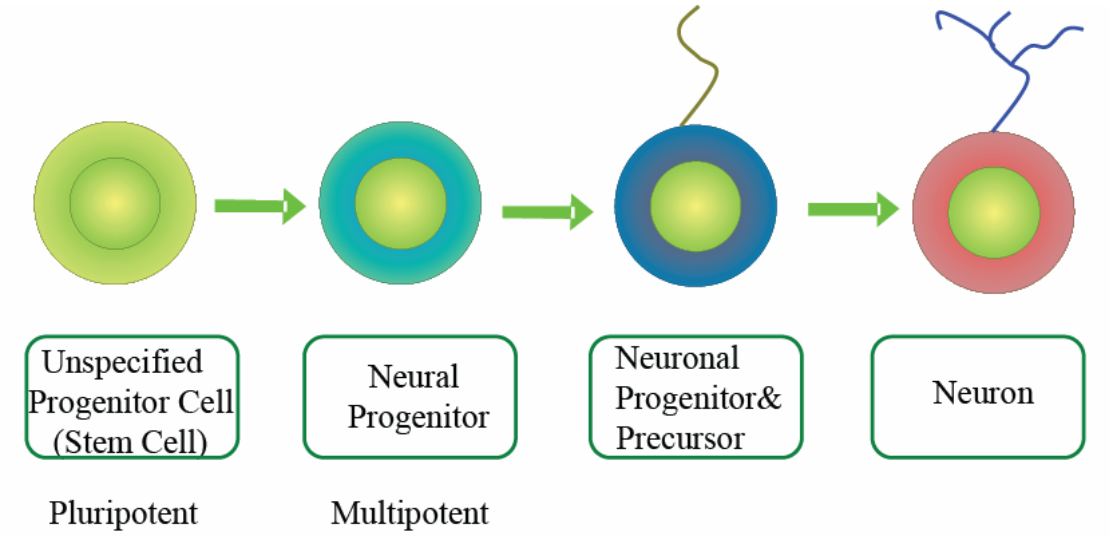

Figure 1.1 The cellular process of neurogenesis. The differentiation of stem cells into mature neurons can be divided into three steps: Neurogenesis starts from the differentiation from unspecified progenitors (pluripotent) into neural progenitors exclusive for the nervous system (multipotent); neural progenitors that contribute to neuronal lineages further differentiate into immature neuronal progenitors and neuronal precursors; Neuronal precursors will subsequently turn into mature neurons.

the differentiation of pluripotent progenitors into multipotent neural progenitors, and neural progenitors have the potentiality to develop into neurons, glial cells, epidermal cells and endothelial cells.[2] The second step is the differentiation of neural progenitors into neuronal progenitors and precursors, which are limited to become neurons. The last step is the maturation of those neuronal cells. However, within the CNS, specific criteria have not been established to define each step, and this model has been challenged by new evidence.[3]

The canonical Wnt signaling pathway in neurogenesis

Each step of neurogenesis has several key regulators including well-known signaling pathways such as Wnt, Notch, BMP, FGF, Shh, etc. The canonical Wnt signaling pathway is a network of proteins first known for their roles in embryonic development.[4] The pathway initiates from the binding of secreted Wnt ligands to Frizzled receptors and LRP5/6 co-receptors on target cells, followed by the recruitment of Axin2 to the cellular membrane and the stabilization of cytoplasm β-catenin, which moves into the nucleus and activates transcription of Wnt target genes.

As one of the most studied developmental regulators, the canonical Wnt signaling pathway has been reported to control both neural induction and neuronal specification, in addition to its role as a general mitogen.[5-7] Wnt signaling has also been proposed to regulate the differentiation of glial cells, and recent evidence suggests that glia can also function as intermediate neuronal progenitors.[8, 9] The reported functions of the canonical

Wnt signaling pathway in neurogenesis are summarized in the introduction parts of Chapter 2 and 3, as well as in Tab. 1.1. As shown in the table, there is conflict in the published literature about the role of Wnt signaling in neurogenesis. Some evidence suggests that Wnt is a neural inducer, while some others suggest Wnt is an inhibitor for neurogenesis. For example, conditional Knock-Out (cKO) of β-catenin in neural stem cells via Nes^{Cre} induces the expression/expansion of intermediate neuronal progenitor markers in cortex, while conditional dominant active β-catenin (β-catenin-σEx3) in midbrain progenitors via Shh^{Cre} also keeps them as intermediate neuronal progenitors.[10, 11]

These inconsistencies are commonly attributed to context-dependent diversity. It is known that the canonical Wnt signaling pathway utilizes various combinations of ligands, receptors, and transcription factors, and that Wnt/beta-catenin/Tcf signal transduction in the cytoplasm and nucleus can avail themselves of multiple protein cofactors.[12, 13] Most importantly, the fact that the canonical Wnt signaling pathway is vital for early embryogenesis also makes it difficult to perform cell type-specific observations, especially when trying to interpret neurogenesis phenotypes from a mispatterned brain sample.[14]

Table 1.1 Reported functions of Wnt signaling in neurogenesis.

Wnt functions in neurogenesis	**Description of evidence**
Morphogenesis	Mouse mutants of required Wnt pathway components like β-catenin or Axin1 may have severe morphologic defects during gastrulation and lead to early embryonic lethality around E9.5.[14, 15] Tcf3 is required for the forebrain formation.[16, 17]
Proliferation	Wnt1 induces expansion of the midbrain progenitor pool.[18] Lef1 and/or LRP6 null mutants have smaller cerebral cortical and hippocampal fields.[19, 20]
Neurogenesis inhibition	Ectopic β-catenin/lef1 fusion protein delays neurogenesis.[21] Inhibition of β-catenin causes premature differentiation of cortical neurons.[22]
Neural induction	Wnt3, Wnt3a, and Wnt5b transiently promote the differentiation of neural progenitors into neuronal and astrocyte lineages.[23-25]
Neuronal specification	Loss of Wnt1 causes loss of dopaminergic neurons and ectopic production of serotoninergic neurons in the ventral midbrain.[26, 27] GSK3β, β-catenin, and Lef1 are involved in GABAergic neuronal differentiation.[28, 29]
Transient role in neurogenesis	Wnt3, Wnt3a, and Wnt5b have been suggested to be transiently required for proliferation and further differentiation into neuronal (Map2t) and astrocyte lineages in neonatal or adult neural progenitor cultures.[23-25]
Complex role in gliogenesis	Traumatic brain injury will increase cell division and β-catenin reporter cells in cortex.[30] Wnt influences the timing and efficiency of oligodendrocyte precursor cell generation in the telencephalon.[31] Wnt is an essential and direct driver of myelin gene expression and myelinogenesis.[32]
Adult neurogenesis	In adult mice, Wnt LOF in neural stem cells by $Sox2^{Cre}$ retrovirus injection results in arrest as GFAP+ radial stem-like cells in the hippocampus.[33] Wnt GOF in radial stem-like cells with APC (Wnt inhibitor) cKO via $GFAP^{Cre}$ are arrested as Mash1(Ascl1)+ transit amplifying cells (TACs).[34]

The vertebrate hypothalamus as a model to study

the Wnt signaling in neurogenesis

To further investigate the role of the canonical Wnt signaling pathway in brain neurogenesis, we used the vertebrate hypothalamus as a novel neurogenic system. Compared with other parts of the forebrain (hippocampus, cortex, and olfactory bulb), the hypothalamus is relatively uninvestigated as a model organ of neurogenesis. The hypothalamus is a highly conserved vertebrate brain structure and is commonly the ventral-most portion of the vertebrate forebrain, with the third ventricle passing through its midline (Fig. 1.2). The hypothalamus is unique in being the only brain structure linking the nervous system and the endocrine system via the pituitary body. As this link, the hypothalamus is primarily responsible for metabolic regulation and physiological activities, and has been found to be involved in feeding, sleeping and mating behavior.[35] Additionally, the hypothalamus is an important component of the limbic system, contributing to more complex functions including learning and memory.[36]

Much has been published to indicate that the hypothalamus has Wnt activity, and cellular proliferation in the adult hypothalamus has been observed in multiple species including the zebrafish and mouse (Fig. 1.3).[37] Using BrdU pulse-chase experiments in the adult zebrafish and adult mouse hypothalamus, various researchers have shown that newly born neurons arise from a progenitor population in as little as seven days after BrdU exposure.[38] However, it is unknown what role Wnt signaling may play during embryonic and adult hypothalamic neurogenesis.

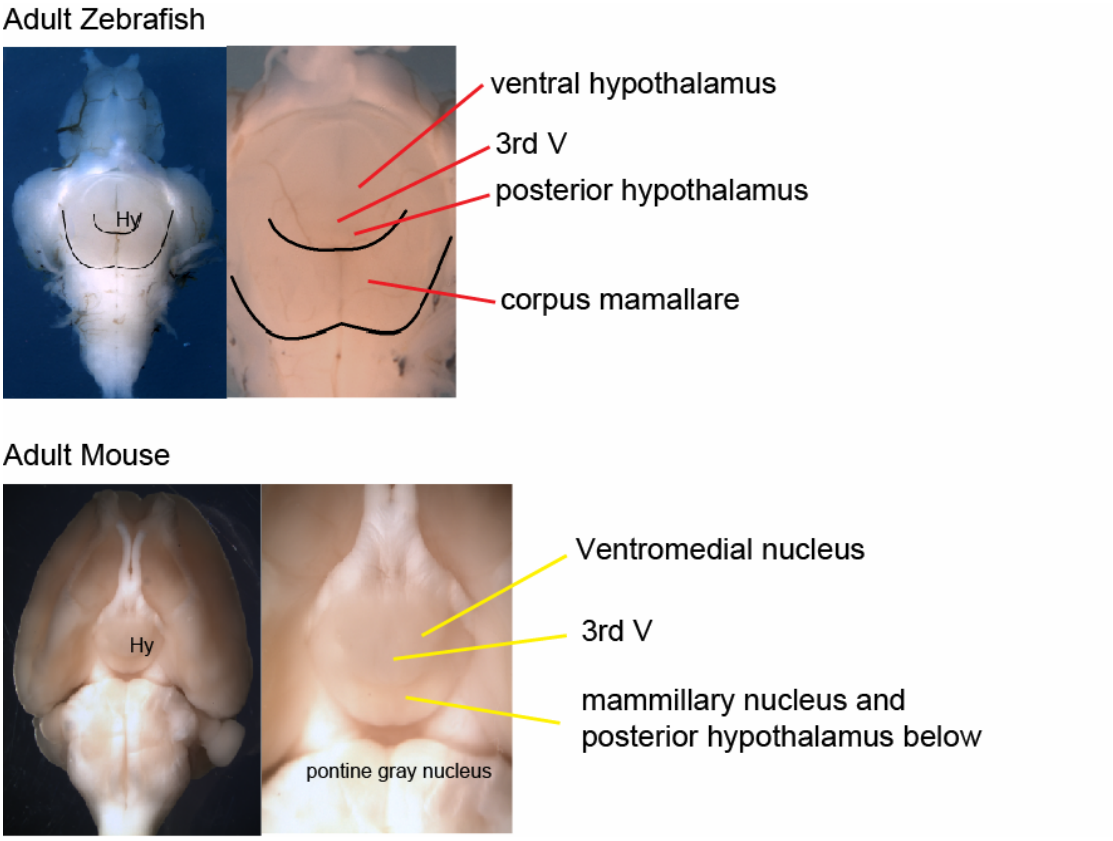

Figure 1.2 The hypothalamus in the adult zebrafish and mouse brains. In zebrafish and mouse, the hypothalamus is located on the ventral surface of the brain with similar morphology and neuroanatomical architecture.

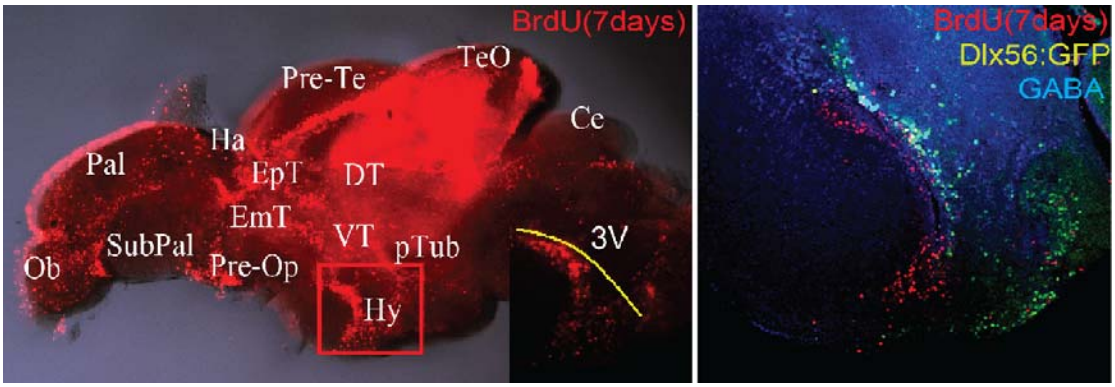

Figure 1.3 7 day BrdU tracing in the adult zebrafish hypothalamus. Animals were sacrificed 1 week after a single BrdU pulse, and staining on sagittally sliced brain suggests that the adult zebrafish hypothalamus maintains proliferation activity (Left). Some BrdU labeled cells are Dlx^{+} GABAergic neurons or precursors (Right).

Our lab first investigated this question using the zebrafish hypothalamus. A previous graduate student, Ji Eun Lee, uncovered the expression of Lef1 in the developing zebrafish hypothalamus and showed that Lef1 may be required for proneural andneuronal gene expression in the posterior hypothalamus using lef1 morpholino knockdown.[29] However, the role of Wnt signaling in the postembryonic hypothalamus was unstudied at the beginning of my thesis work.

My project aimed to perform a new series of analyses on both embryonic and adult neurogenesis in the hypothalamus, to determine the answers to three basic questions: 1) which Wnt components contribute to hypothalamic Wnt activity; 2) what cell types in the hypothalamus are Wnt-responsive; 3) what is the function of Wnt signaling in embryonic and adult hypothalamic neurogenesis. This thesis addresses some of the controversies in the field by focusing on a single area of the brain at different developmental stages in different species, thereby uncovering the potentially dynamic nature in the role of Wnt signaling in neurogenesis.

Anatomy of the zebrafish and adult mouse hypothalamus

The zebrafish hypothalamus is initially formed at the end of gastrulation, when the rostral diencephalic ventral midline cells begin to protrude caudally.[39] The budding hypothalamus maintains its "neural tube" shape (Fig. 1.4) until about 36 hours postfertilization (hpf), at which point the posterior hypothalamus around the posterior recess of the third ventricle elongates laterally, forming an inverted "T" ventricular

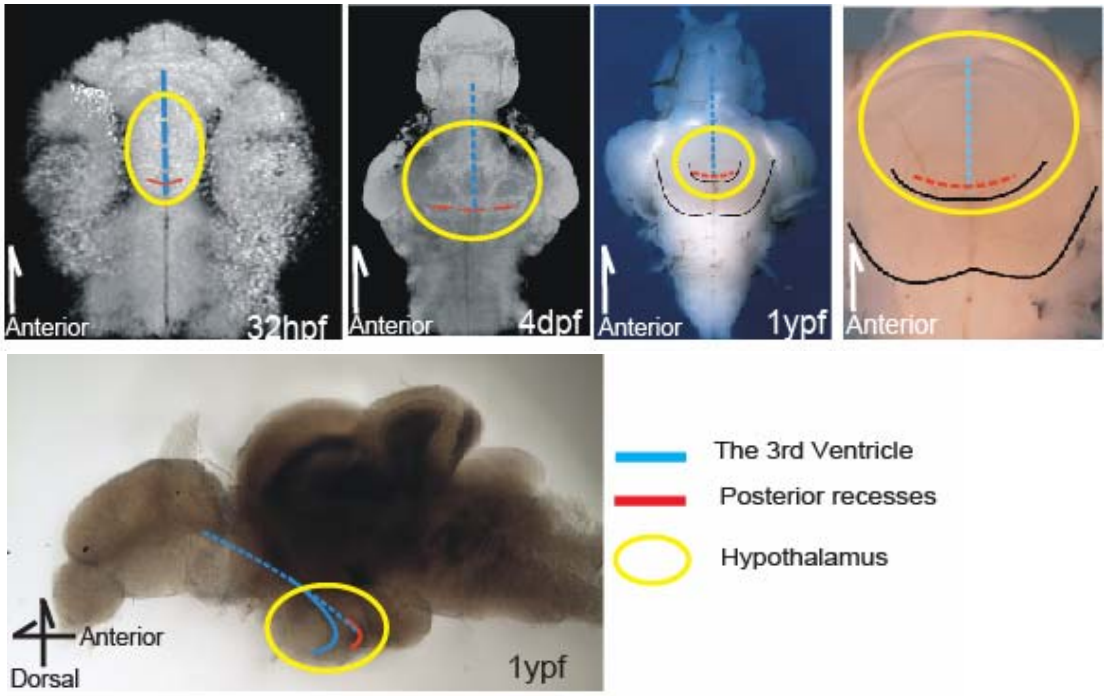

Figure 1.4 The anatomy of embryonic and adult zebrafish hypothalamus. At early stages (32hpf), the zebrafish hypothalamus is simply shaped as a second neural tube beneath the primary neural tube. At later embryonic stages (4dpf), the protrusions of the 3rd ventricle in the medial and posterior hypothalamus undergo dramatic changes in morphology, which are maintained into the adulthood. The ventricular system of the zebrafish hypothalamus serves as a reliable landmark.

system. The third ventricle and its elongated posterior recess serve as reliable hypothalamic landmarks into adulthood, and the overall anatomy of adult hypothalamus is almost identical to the 4 days postfertilization (dpf) hypothalamus, except for being surrounded by larger caudal protrusions from the inferior lobe of the posterior tubercle (Fig. 1.4). From the sagittal slice of the adult zebrafish brain, we can also observe a lateral recess projecting from the 3rd ventricle, which is formed at approximately the same time as the posterior recess.

Due to the size of the adult mouse hypothalamus, it is impossible to observe it as a whole via confocal microscopy. By examining coronal sections of an entire adult mouse hypothalamus, I found that the third ventricle can also be used as a landmark (Fig. 1.5). Unlike the zebrafish hypothalamus, there is no lateral recess or posterior recess in the adult mouse hypothalamus, and the mouse hypothalamus can be divided into several well-defined nuclei (Fig. 1.5). Comparison of zebrafish and mouse hypothalamus anatomy suggests high conservation through evolution, and also leads to the question of whether expression patterns of Wnt components are conserved at different times in different organisms. This thesis will demonstrate a detailed genetic atlas for the Wnt signaling pathway in the zebrafish and mouse hypothalamus.

Neurogenesis in the zebrafish hypothalamus

The zebrafish hypothalamus contains several different neuronal lineages derived from neurogenic niches. At early embryonic stages (32hpf), progenitor cells expressing

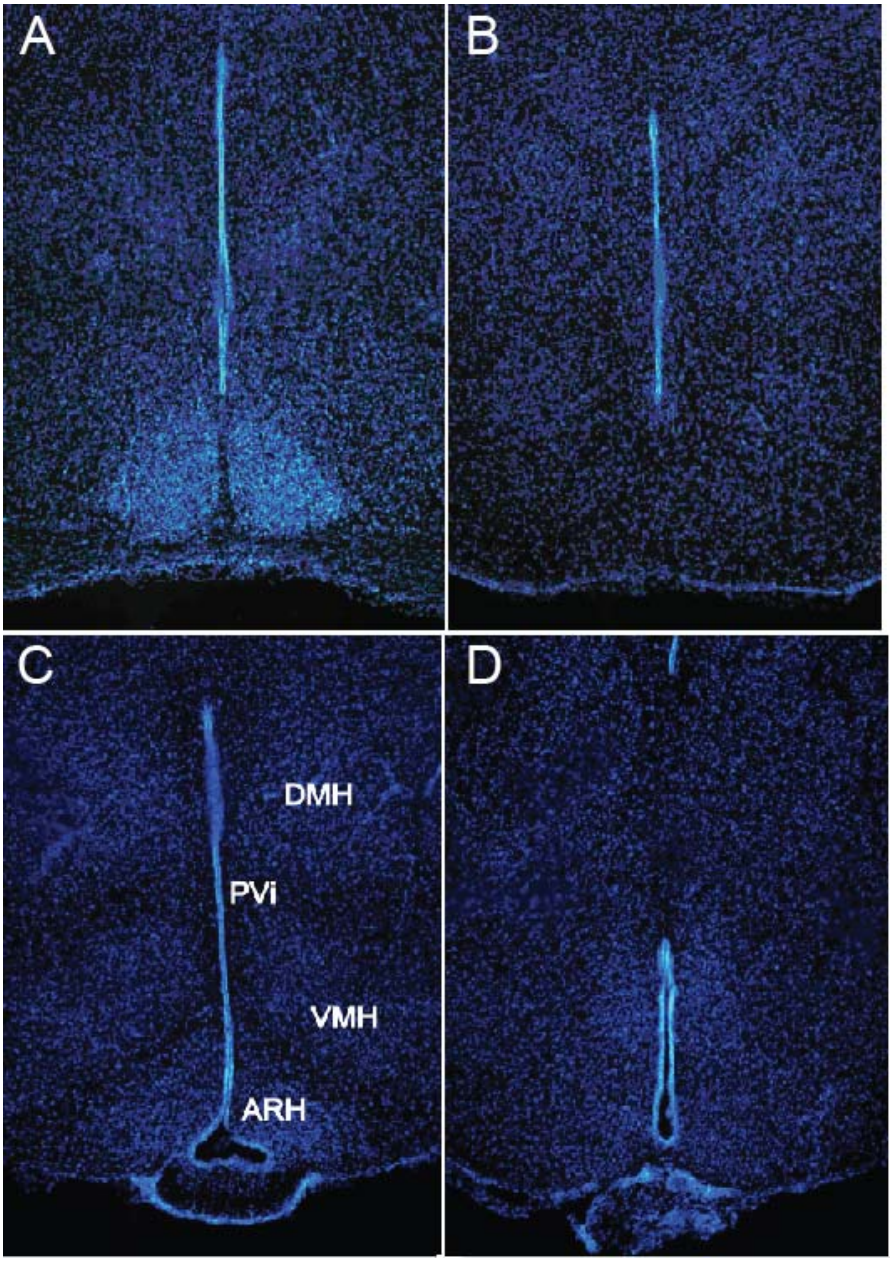

Figure 1.5 Nuclear staining of adult mouse hypothalamic coronal sections (A-D: Anterior to posterior)

DMH: dorsal middle hypothalamic nucleus; PVi: paraventricular hypothalamic nucleus; VMH: ventral middle hypothalamic nucleus; ARH: arcuate hypothalamic nucleus.

PCNA, a marker of proliferation, fill the majority of the hypothalamus. In contrast, cells positive for Sox3, a neural progenitor marker, are found only in the mid-hypothalamus, the boundary region of the anterior hypothalamus, and the pre-optic region (Fig. 1.6). Using *dlx5/6* enhancer expression as a marker of GABAergic neuronal precursors, we find *dlx5/6*-positive cells only in the marginal zone of the hypothalamus (Fig. 1.6). In other words, the 32hpf hypothalamus is filled with progenitors, of which only a small proportion are specified neural progenitors. At later embryonic stages (4dpf), the expression of PCNA becomes more restricted to the ventricular region, while the Sox3-positive neural progenitor pool expands across the entire hypothalamus (Fig. 1.6). Two additional neural progenitor markers, *sox2* and *ascl1a*, are also expressed in the ventricular regions, but at different levels (Fig. 1.6). Additionally, both *dlx5/6* transgene expression and *dlx2* in-situ hybridization suggest that there are more neuronal precursors in the zebrafish hypothalamus at this stage than at 32hpf (Fig. 1.6). All progenitor and precursor markers shown in the 4dpf zebrafish hypothalamus maintain similar expression patterns into adulthood (data not shown).

HuC/D and acetylated α-tubulin staining suggest that there are postmitotic neurons born in the zebrafish hypothalamus as early as 32hpf (Fig. 1.7). The first identified neuronal subtype is the GABAergic neuron, which is located in the anterior marginal region of the hypothalamus. At 4dpf, there are other neuronal lineages emerging besides the broadly distributed GABAergic neurons, including dopaminergic and serotonergic cells (Fig. 1.7). In-situ hybridization analyses suggest that GABAergic neurons in the

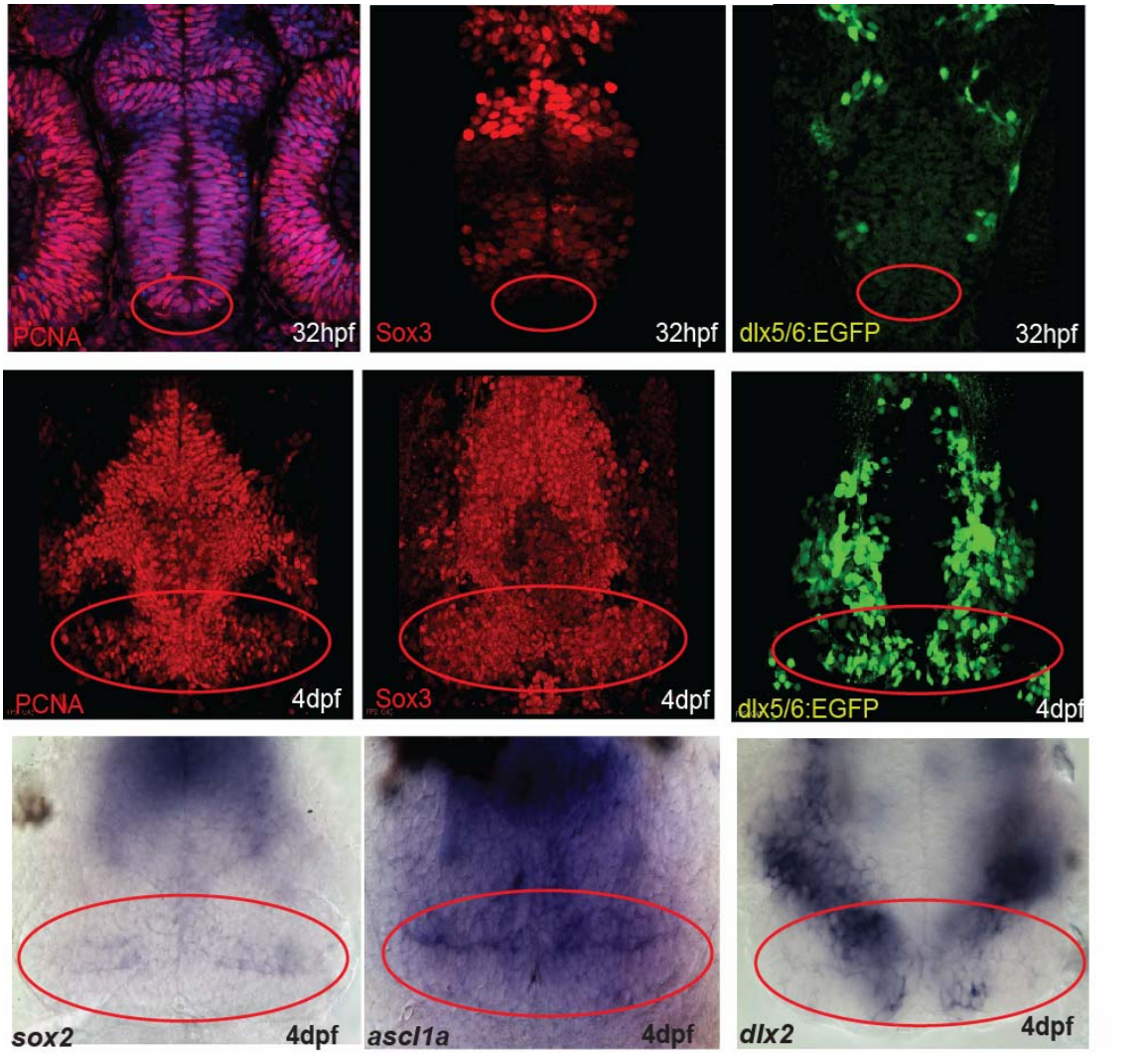

Figure 1.6 Neural progenitors and precursors in the zebrafish hypothalamus. Red circles indicate posterior hypothalamic regions. PCNA is a general proliferation marker and labels progenitors; Sox3 is a neural progenitor marker; *dlx5/6:EGFP* labels the majority of GABAergic neuronal precursors; *sox2* is another neural progenitor marker expressed earlier than Sox3, *ascl1a* is another neural progenitor marker expressed later than Sox3; *dlx2* is also a neural progenitor marker expressed later than *ascl1a*, but earlier than *dlx5/6*.

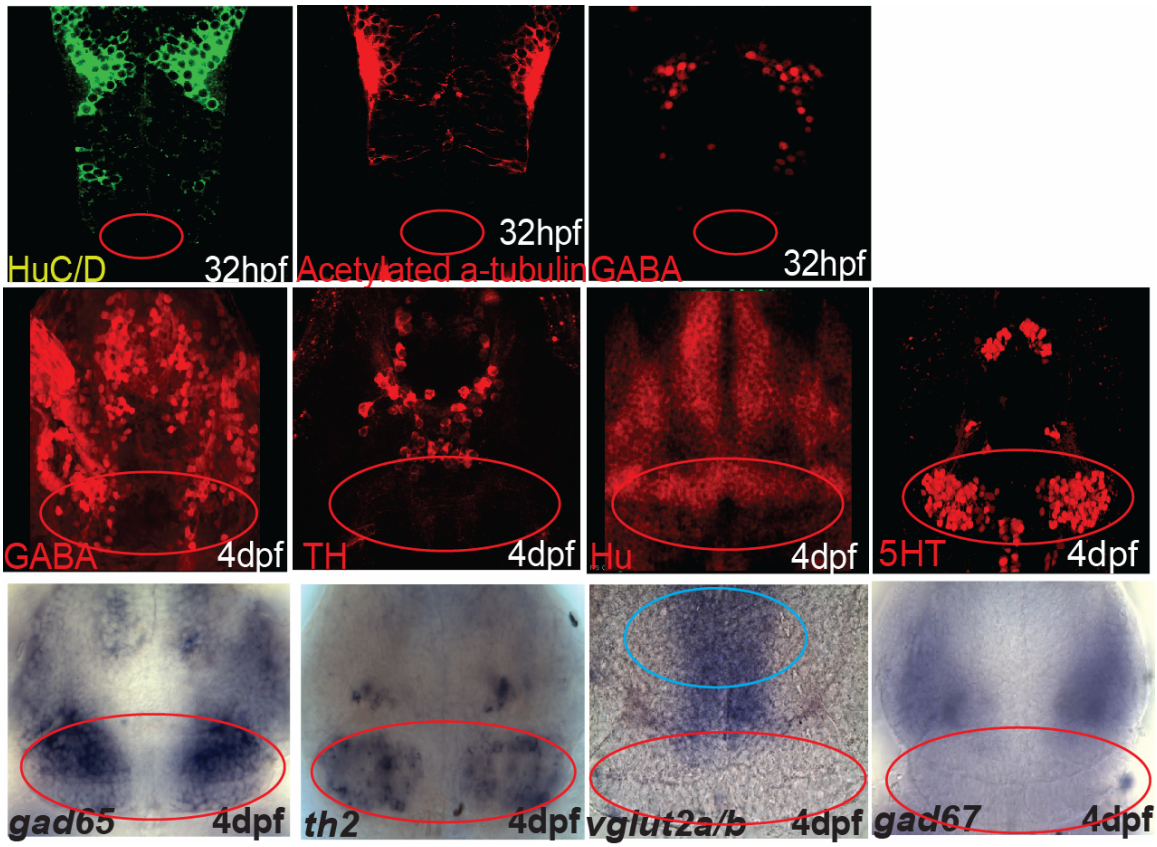

Figure 1.7 Neurons in the zebrafish hypothalamus. HuC/D is a ubiquitous neuronal marker; acetylated alpha-tubulin is a marker for neurites as well as neurons; GABA, TH, and 5HT stain GABAergic neurons, dopaminergic neurons, and serotonergic neurons, respectively; *gad65* and *gad67* encode glutamate decarboxylases expressed in GABAergic precursors; *th2* is one of the two tyrosine hydroxylase genes expressed in zebrafish; *vglut2a/b* encodes a vesicular glutamate transporter expressed in a subset of excitatory neurons.

posterior recess region may be express *gad65* but not *gad67*, and *th2* may label a dopaminergic population in the posterior hypothalamus that is not labeled by any TH antibody (Fig. 1.7). Also, *vglut2a/b*-expressing glutamatergic neurons only exist in the anterior medial hypothalamus (Fig. 1.7). All these neuronal subtypes have also been identified in the adult zebrafish hypothalamus (data not shown).

Why hypothalamic neurogenesis is important

As mentioned above, the hypothalamus regulates a wide range of physiological and behavioral activities. To realize these functions, the hypothalamus exchanges information with many different parts of the brain, and itself plays a major role in the hypothalamic-pituitary-adrenal (HPA) axis. Several medical conditions have been determined to be associated with dysfunction of the hypothalamus including self-mutilation, depression, schizophrenia and bipolar disorder, and it has been found that treatment with antidepressants reduces HPA activity.[40, 41] The modification of hypothalamus activity or HPA activity will have significant medical value.

However, we know little about how to modify a normal hypothalamus or to cure a dysfunctional hypothalamus, and the studies of hypothalamic neurogenesis will supply us with potential mechanisms. One principal function of dopamine in the hypothalamus is to inhibit the release of prolactin from the anterior lobe of the pituitary.[42] while dopaminergic and serotonergic neural systems in the lateral hypothalamic area and the ventromedial nucleus regulate food intake including the meal size and meal frequency.[43]

GABA is the dominant inhibitory neurotransmitter in the hypothalamus, and the interactions between the GABAergic and dopaminergic neural systems in the lateral anterior hypothalamus are associated with the aggression control and stress-induced inhibition.[44] If we can decipher the actual neurogenesis process and innervation of these neural systems, we may be able to modify neuronal populations to cure hypothalamic dysfunction or to achieve other goals like food intake control or mood control.

As mentioned above, we have identified several neuronal lineages in the zebrafish hypothalamus, including GABAergic, dopaminergic, and serotonergic lineages, whose potential functions have just been described. However, the role of Wnt signaling in the neurogenesis of each lineage is not known. This thesis addresses this question in the following chapters.

Overview of Chapters 2 and 3

Chapter 2 of this thesis is presented in two parts: a review of previously published research about "Wnt and Neurogenesis," and partly as a research article focusing on the expression of Wnt components in the zebrafish hypothalamus. The research article highlights two major observations: (1) a Wnt reporter and multiple Wnt signaling pathway members are expressed in the embryonic and adult zebrafish hypothalamus; (2) Wnt-responsive cells in the zebrafish hypothalamus are intermediate neural progenitors.

Chapter 3 of this thesis focuses on the functions of Wnt activity in the zebrafish hypothalamus and their conservation in the adult mouse hypothalamus. The first major

finding described is the observation that hypothalamic Wnt activity fulfills different roles in the course of neurogenesis at different stages of development. During early embryogenesis, Wnt signaling functions as a general mitogen. Starting at 4dpf and continuing into adulthood, Wnt signaling is not critically required for proliferation; instead, it is transiently required for neuronal differentiation. The second major finding described is that the adult mouse hypothalamus also has a Wnt-responsive population that may contribute to adult neurogenesis, similar to observations from the adult zebrafish hypothalamus. Although we found that ventricular $Hes1^{+}$ neural progenitors do not give rise to newborn neurons in the adult mouse hypothalamus, we found that Wnt inhibits the differentiation of hypothalamic neural progenitors into tanycytes, a role that is conserved between zebrafish and mouse.

Taken together, by analyzing both the adult mouse hypothalamus and the zebrafish hypothalamus at several developmental stages, I have established a new model in which to study neurogenesis, and have elucidated the dynamic roles of Wnt signaling, which will be explained in detail in Chapters 2 and 3.

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CHAPTER 2

IDENTIFICATION OF WNT-RESPONSIVE CELLS IN THE ZEBRAFISH HYPOTHALAMUS

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Wnt Signaling

Identification of Wnt-Responsive Cells in the Zebrafish Hypothalamus

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Abstract

In all vertebrate brains, there is a period of widespread embryonic neurogenesis followed by specific regional neurogenesis that continues into adult stages. The Wnt signaling pathway, which is essential for numerous developmental processes, has also been suggested to be involved in neurogenesis. To help investigate the exact roles of canonical Wnt signaling in neurogenesis, here we examine the identity of Wnt-responsive cells in the zebrafish hypothalamus. This tissue is a useful diencephalic neurogenesis model containing evolutionarily conserved populations of neurons. We first performed *in situ* hybridization to show the expression patterns of Tcf family members and a canonical Wnt signaling reporter in the 50 hpf embryonic hypothalamus and larval/adult hypothalamus. We then used immunohistochemistry to identify the cell types of Wnt-responsive and Lef1-positive cells in both 50 hpf embryonic and adult hypothalamus. Our results indicate that Wnt-responsive cells in the hypothalamus are likely to be both mitotic progenitors and postmitotic precursors at embryonic stages, but only precursors at the adult stage. These data suggest that canonical Wnt signaling may be functionally required for maintenance of neural progenitor and precursor pools in the embryo, and for ongoing neurogenesis in the adult zebrafish.

Introduction and Review

THE WNT SIGNALING PATHWAY regulates early patterning, morphogenesis, and cellular function throughout the animal kingdom, with evolutionary conservation between vertebrates and invertebrates. The canonical Wnt pathway involves a signaling cascade that ultimately results in the stabilization of β-catenin, which is translocated into the nucleus, where it binds to Lef/Tcf high mobility group (HMG) transcription factors and activates transcription.[1] Previous reviews have summarized how Wnt signaling generally regulates embryonic development, and the roles of Wnt signaling in neural development.[2] Here we will emphasize the roles of Wnt signaling in vertebrate brain neurogenesis.

Although the roles of Wnt signaling in early embryonic patterning are relatively well understood, the function of this pathway in neurogenesis is less clear. Neurogenesis at the cellular level consists of three distinct processes: (1) proliferation and cell cycle exit, (2) proneural specification, and (3) neuronal (and glial) differentiation. Identification of canonical Wnt pathway targets supports roles in all of these processes. For example, *Cyclin D1*, *Cdx4*, *Neurogenin1*, and *Sox3* are all identified direct transcriptional targets.[3–6] The first two genes encode regulators of cell proliferation, while the second two encode regulators of proneural specification. In addition, canonical Wnt signaling has been suggested to regulate multiple neuronal/glial differentiation factors.[3,7] However, it is not clear whether Wnt signaling plays consistently positive or negative roles in any of these processes.

Conflicting roles for Wnt signaling in neurogenesis

Most published data support the idea that the Wnt signaling pathway promotes neural progenitor proliferation. At the ligand level, Wnt1 acts as a mitogen and an apoptosis inhibitor in the developing CNS. Ectopic Wnt1 can induce overproliferation of caudal midbrain via expansion of the progenitor pool, which may be attributed to the shorter cell cycle length and decreased cell cycle exit.[8–11] Wnt3a, Wnt7a, Wnt7b, and Wnt10b also stimulate the proliferation of neural progenitors. In mouse embryos lacking Wnt3a, a total loss of hippocampus and reduction of caudomedial cerebral cortex are observed, as the hypothesized result of progenitor proliferation defects,[12,13] and zebrafish embryos lacking Wnt3a, Wnt1, and Wnt10b undergo extensive apoptosis in the midbrain and cerebellum.[14] Similarly, in Lef1 null mutants, LRP6

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mutants, and D6-Cre lines with conditional inactivation of β-catenin or activation of Dkk1 (a secreted Wnt inhibitor) in the mouse cerebral cortex, the hippocampal fields are significantly reduced in size.[7,15–18] Specifically, neurogenesis in the dentate gyrus is decreased in both the premigratory and migratory progenitor pools, and LRP6 null mutants with a single Lef1 null allele exhibit more severe defects, as do embryos expressing a Lef1-lacZ fusion gene, which encodes a dominant-negative protein that blocks all Lef/Tcf function.[15,17] Finally, β-catenin has been shown to be essential for maintenance and proliferation of neural progenitors, as it can promote proliferation of Mash1+progenitor cells in the subventricular zone, and the overexpression of β-catenin can induce a larger forebrain with increased neuronal production.[19–21] Together, these data suggest that Wnt signaling promotes neurogenesis primarily by increasing the size of the progenitor pool.

Other studies suggest that Wnt activity must be downregulated for progenitors to differentiate. Ectopic expression of a β-catenin/Lef1 fusion protein delays the expression onset of neural markers and subsequent neurogenesis, and conditional ablation of β-catenin can also accelerate expression of some neural markers.[22] Targeted inhibition of β-catenin signaling during embryonic development causes cortical progenitor cells to prematurely differentiate into neurons and migrate to the cortex.[23] However, these findings are contradicted by other studies showing that Wnt activity directly leads to the specification of neural progenitors. In *Xenopus*, ectopic expression of mouse Wnt8, X-Wnt8, β-catenin, or dominant-negative glycogen synthase kinase 3 (GSK3) induces the expression of neural marker NCAM.[24] In zebrafish, the canonical Wnt signaling pathway via Wnt8b and Lef1 has been suggested to be involved in posterior hypothalamic neural specification without significant effects on proliferation and apoptosis.[4] In addition, Wnt3 is expressed in the hippocampal neurogenic niche, and overexpression of Wnt3 increases neurogenesis.[25] These opposite roles of Wnt signaling during neurogenesis have been attributed to differences in context between the relevant cell populations.

Most *in vitro* experiments on cultured embryonic stem cells (ESCs) support the idea that Wnt signaling contributes to the maintenance of embryonic stemness while inhibiting neural differentiation. Forced expression of Wnt1, Wnt5a, or Wnt6 inhibits neural differentiation from ESCs.[26,27] Manipulations that can inhibit the canonical Wnt signaling pathway, such as Sfrp2 treatment and Dkk1 induction, can stimulate neural differentiation from ESCs.[26,28] Manipulations that can activate the canonical Wnt signaling pathway, such as lithium chloride treatment, adenomatous polyposis coli inactivation, and expression of a dominant active form of β-catenin, all inhibit neural differentiation.[26,29] In addition, 6-bromoindirubin-3-oxime, a specific pharmacological inhibitor of GSK3, maintains the pluripotent state of ESCs indicated by the expression of ESC markers like Oct3/4, Rex1, and Nanog.[30] By contrast, most *in vitro* experiments using neural progenitor/precursor cell culture support the idea that Wnt signaling contributes to both the proliferation of neural progenitor cells and further neural differentiation. Wnt3, Wnt3a, and Wnt5b have been suggested to be transiently required for proliferation and further differentiation into neuronal (Map2+) and astrocyte lineages in neonatal or adult neural progenitor cultures.[31–33]

It is possible that the Wnt signaling pathway plays different roles in nonneuralized stem cells and neural progenitors or precursors. Before stem cells or their progeny undergo neural specification, Wnt signaling could inhibit neurogenesis, while Wnt signaling could promote further differentiation in neural progenitors or precursors. In both situations, Wnt signaling seems to promote proliferation. The conflicting results obtained from *in vivo* experiments could be attributed to different composition of the tested tissues, as it is expected that these tissues usually contain both nonneural and neural progenitors. The relative proportion of each progenitor subtype might depend on how mature those tissues are.

The Wnt signaling pathway plays roles in neuronal and glial differentiation

Several studies have also suggested a role for the Wnt pathway in neuronal subtype differentiation. It has been shown that Wnt signaling is important for dopaminergic and GABAergic neuronal development at different contexts. Wnt1 acts to specify the midbrain-dopaminergic (mDA) precursors in mouse embryos. Loss of Wnt1 causes loss of mDA neurons and ectopic production of 5-HT serotoninergic neurons in the ventral midbrain.[34,35] Wnt1 and Wnt3a can promote the proliferation of Nurr1+mDA precursors, while Wnt5a functions in the transition from Nurr1+mDA precursors to tyrosine hydroxylase (TH)–expressing mDA neurons.[36,37] However, in zebrafish embryos, dopaminergic precursor number is restricted by the canonical Wnt signaling pathway (Wnt8a, Fz8a, and Lef1) in the diencephalon, which may again reflect a context-dependent difference.[38] Three components of the canonical Wnt signaling pathway—GSK3β, β-catenin, and Lef1—are also involved in GAD67 regulation,[39] suggesting their roles in GABAergic neuronal differentiation. The Wnt pathway also has specific effects on glial subtype differentiation. In the mouse cortex with D6-Cre–driven conditional inactivation of β-catenin, premature disassembly of the radial glial scaffold and increased numbers of astrocytes are found at newborn stages.[16] Activation of Wnt signaling *in vitro* is also able to increase the number of GFAP-positive astrocytes but suppresses the number of oligodendroglial lineage cells labeled by PDGFR or O4.[32]

Mechanisms underlying the differential response to Wnt signals

Several potential mechanisms could explain how neural progenitors and precursors respond differently to Wnt signaling. One possibility is that crosstalk between Wnt and other signaling pathways such as Fgf, Shh, and RA can result in different outputs. Overexpression of β-catenin in the presence of FGF2 helps to maintain neural progenitor cells in a proliferative state, while overexpression of β-catenin in the absence of FGF2 enhances neuronal differentiation.[40] Similarly, Wnt7a and Wnt7b have been shown to differentially regulate proliferation or maturation of neural precursors depending on the context of FGF2 and Shh signaling.[3,13,41] Further, Wnt and FGF signaling may act simultaneously on the promoters of downstream targets such as *Sox2* and can together contribute to the specification of dorsal telencephalic character.[42,43] Retinoic acid (RA) is another signal that can change the response of cells to Wnt signals. Wnt1 induces neuronal differentiation in the absence of RA but inhibits

neural differentiation in response to RA treatment.[26,44] In addition, the Notch intracellular domain can function as a coactivator of Lef1,[45,46] and the BMP signaling pathway can interact with Wnt signaling to regulate neural tube proliferation and patterning.[29,47,48] Recently, epigenetic research also shows that chromatin modification status can affect the selective promoter occupancy by Tcfs.[49] Taken together, these findings suggest that Wnt responses are very context dependent.

Multiple Tcf family members may contribute to the different responses to Wnt signaling

Another level of cell-intrinsic differences is determined by the expression of diverse Tcf/Lef family members. There are four closely related Tcf family members identified in human and mouse[50]—Lef1, Tcf7 (Tcf1), Tcf7l1 (Tcf3), and Tcf7l2 (Tcf4). One of these factors (Tcf7l1) is duplicated in zebrafish, leading to five proteins in total. All Tcf/Lef proteins have a highly similar HMG box that allows specific DNA binding. Biochemical assays using biotinylated oligonucleotides and the HMG domain of Tcf4 have calculated a binding affinity matrix with CCTTTGATG as the highest affinity sequence.[51] Although all Tcf family members contain similar domains, alternative splicing and promoter usage may produce dominant-negative isoforms, contributing to functional diversity.[52] For example, Lef1 is normally a β-catenin–dependent transcriptional activator, but a truncated form of Lef1 lacking the β-catenin binding domain can be produced in colon cancer and lymphocyte development.[53–55] In addition, the cysteine-rich domain of Lef1 can be alternatively spliced in response to TGF-β signaling.[56]

Among the four Tcfs, Tcf7 and Lef1 are highly functionally redundant. Both factors show redundancy in paraxial mesoderm and ectoderm morphogenesis as well as limb development.[57–59] However, more careful investigation suggests that the two factors are not completely interchangeable and have distinct responses to Wnt signaling.[58,60] Partial functional redundancy also exists between Tcf7 and Tcf7l2,[61] which both have dual functions as repressors and activators.[62–64] Tcf7 and Tcf7l2 also share similar C-termini that can cooperate with β-catenin and p300 to form a specialized transcription factor complex for the *Cdx1* promoter, unlike Lef1.[65,66] However, each factor also plays some distinct roles; for example, Tcf7l2 has been suggested to be specifically essential for intestinal development and cancer.[67–69]

By contrast, Tcf7l1 appears to function complimentarily to other Lef/Tcf factors, acting primarily as a repressor. Tcf7l1 is expressed broadly in the early embryo and contributes to tissue patterning together with other Tcfs.[58,60,70–72] Although Tcf7l1 and Lef1 are usually expressed adjacently, they perform distinct functions. In hair follicles, Lef1 promotes the differentiation of hair-producing progenitors, whereas Tcf7l1 may maintain bulge stem cells. The two factors also cooperate in early embryonic ectoderm differentiation and medial pallium development in the telencephalon.[15,60,73] It is likely that the combination of the activator and repressor forms of Tcfs determines the cellular response. Recently, with the development of ChIP-sequencing techniques, Tcf7l1 has been identified as a key factor together with Oct4, Sox2, and Nanog, in contributing to the core regulatory circuitry within ESCs.[74–78]

The zebrafish hypothalamus is a good model to investigate Tcf functions

We are focusing on Wnt-dependent neurogenesis in the zebrafish, using Tcfs as a means to investigate the canonical Wnt signaling pathway. This approach has several advantages. First, the relatively small number of Tcfs compared with 21 identified vertebrate Wnt ligands allows loss-of-function analyses. In addition, these factors act cell autonomously as opposed to secreted Wnt ligands, allowing us to analyze the phenotypes of single cells from a defined population. Finally, Tcf-mediated transcription is the final step of the canonical Wnt signaling pathway, allowing us to analyze a single output and use biochemical tools. In zebrafish, the expression and function of multiple Tcf factors have been examined in detail. In the CNS, canonical Wnt signaling through Lef1 is required for hypothalamic neurogenesis.[4,79] *tcf7* Is specifically expressed in the early dorsal retina and forebrain, but does not alone appear to have a required role in embryonic development. Together with *lef1*, *tcf7* is required for fin and ectoderm development.[59,80] *tcf7l2* is alternatively spliced in zebrafish and exhibits transient expression in the embryonic telencephalon and midbrain,[81] but little is known about its function. *tcf7l1a* and *tcf7l1b* share similar expression patterns and contribute redundantly to early A-P brain patterning.[82,83]

In the mouse, most studies of neurogenesis are performed in the hippocampus, for which there are no comparable structures in the zebrafish embryo. Further, in zebrafish most Tcf family members are absent from the telencephalon after early embryogenesis. We have chosen the hypothalamus as a model to investigate the role of Tcfs in neurogenesis. Previous publications and preliminary work have suggested the expression of transgenic β-catenin–dependent reporters and canonical Wnt components in the embryonic and adult hypothalamus.[4,84,85] In addition, both the embryonic and adult hypothalamus contain a diversity of neurons, glial cells, and proliferating cells.[86,87] Despite these facts, the hypothalamus has been poorly exploited as a model for neurogenesis. It is an evolutionarily conserved endocrine and autonomic organ in vertebrates, and it has recently been suggested that both the vertebrate hypothalamus and the insect pars intercerebralis trace back to a simple brain with neurosecretory cells that existed in common bilaterian ancestors.[88,89] The anatomical and functional conservation of the hypothalamus between diverse vertebrate species makes it an attractive system for uncovering mechanisms of neurogenesis in model organisms. While the role of Wnt signaling in early patterning of the embryonic hypothalamus has been well studied in zebrafish,[90] the function of this pathway in later neurogenesis is poorly understood. Here we examine the identity of TCF-expressing and Wnt-responsive cells in the embryonic, larval, and adult hypothalamus, as an initial step leading to further functional studies.

Materials and Methods

Zebrafish

Embryos were obtained from natural spawning of wild-type (*AB**), *Tg(TOP:dGFP)*^{w25}, and *Tg(1.4dlx5a-dlx6a:GFP)*^{ot1} zebrafish lines.[91,92] Adult brains were dissected from anesthetized adult fish fixed in 4% paraformaldehyde for 2 days.

In situ *hybridization and immunohistochemistry*

Probe synthesis and *in situ* hybridization were performed as described elsewhere.[93] The following RNA probes were used: *lef1*,[79] *tcf7*,[80] *tcf7l1a/b*,[82] *tcf7l2*,[81] *gfp*,[91] and *axin2* (made in our laboratory).

Antibodies and their working dilution ratios are listed below:

Rabbit-anti-GFP (Molecular Probes [Carlsbad, CA], A11122, 1:500)
Mouse-anti-GFP (Molecular Probes, A11120, 1:250)
Affinity-purified rabbit anti-Lef1 (Open Biosystems [Huntsville, AL], 1:500)
Rabbit-anti-GABA (Sigma [St. Louis, MO], A2052, 1:500)
Rabbit-anti-5HT (ImmunoStar [Hudson, WI], Part 20080, 1:1000)
Mouse-anti-TH (ImmunoStar, Part 22941, 1:500)
Mouse-anti-Hu (Molecular Probes, A21271, 1:500)
Mouse-anti-PCNA (Sigma, P8825, 1:1000)
Mouse-anti-GFAP (zrf-1; Zebrafish International Resource Center [Eugene, OR], 1:1000)
TO-PRO-3 iodide (Molecular Probes, T3605, 1:1500)

For whole-mount immunostaining, embryos were fixed with 4% paraformaldehyde for 3 h at room temperature, and incubated with primary and secondary antibodies at 4°C overnight. For whole-mount photography after all staining methods, yolks and eyes of embryos were dissected.

Sectioning and microscopy

Cryosections were cut at a thickness of 12 μm for embryos and 25 μm for adults. Plastic sections were cut at a thickness of 10 μm. Fluorescent images of whole-mount embryos and cryosections were taken using an Olympus FV1000 confocal microscope and a fluorescent dissecting microscope. Bright-field images were obtained using a conventional compound microscope.

Results

The embryonic hypothalamus contains Wnt-responsive cells

Although Tcfs and the Wnt reporter TOP:dGFP have been shown to be expressed in the brain during early zebrafish embryogenesis,[4,79–82] expression after 40 hpf has not been characterized. To assess the precise expression patterns of these markers in the late embryonic hypothalamus, we analyzed embryos at 50 hpf. We chose this stage because the hypothalamus is anatomically distinct and contains dividing progenitors as well as postmitotic precursors and multiple differentiated cell types.

At 50 hpf, *lef1* is expressed strongly in the tectum opticum (TeO), habenula (Ha), and the posterior hypothalamus (Fig. 1A). In the hypothalamus, *lef1* expression can be observed around the presumptive posterior recess of the diencephalic ventricle (Fig. 1A). *tcf7* Is also expressed in the posterior hypothalamus, around the presumptive posterior recess of the diencephalic ventricle (Fig. 1B). Cross sections through the posterior hypothalamus show that *lef1* and *tcf7* are expressed most strongly in the marginal regions, where postmitotic precursors and neurons reside (Fig. 1G, H). At 50 hpf, *tcf7l1a* and *tcf7l1b* are both expressed at low levels throughout the posterior hypothalamus (Fig. 1C, D). Cross sections through the posterior hypothalamus show that *tcf7l1a* is expressed more broadly, whereas *tcf7l1b* is expressed primarily in the medial region adjacent to the ventricle, where neural progenitors/stem cells reside (Fig. 1I, J). By contrast, *tcf7l2* expression is almost absent from the entire ventral diencephalon (Fig. 1E, K). At 50 hpf, the only expression of *tcf7l2* in the hypothalamus is found right above the future hypophysis (Fig. 1E), consistent with the finding that Tcf7l2 negatively regulates pituitary growth in early mouse embryos.[94] Expression of the canonical Wnt reporter *top:dgfp*,[91] as detected by *in situ* hybridization for *gfp* mRNA, is found throughout the hypothalamus at 50 hpf (Fig. 1F, L), suggesting the presence of canonical Wnt activity in a large region. Considering the requirement of *tcf* gene function for transcription of the *gfp* reporter, broad expression of *gfp* may reflect the combined expression of multiple Lef/Tcf factors earlier in development.

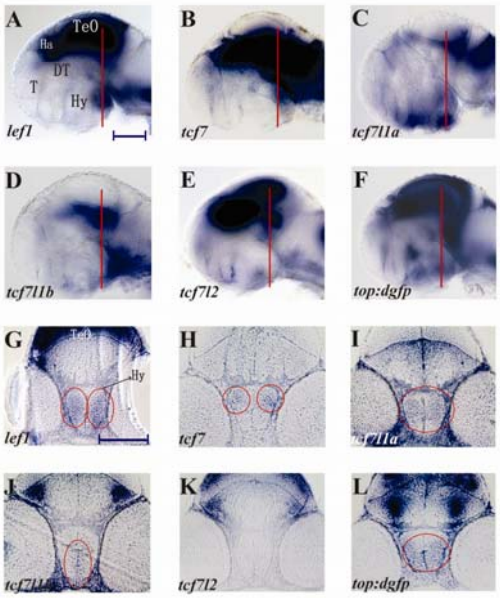

FIG. 1. Expression of *tcf* and *top:dgfp* mRNA in the 50 hpf embryonic zebrafish hypothalamus. (**A–F**) Lateral views of whole-mount brains at 50 hpf. (**G–L**) Ten-micron plastic cross sections of the posterior hypothalamus. Sectioned regions are indicated in panels (**A–F**). Circled regions in (**G–L**) indicate domains of specific gene expression. *top:dgfp* and all *tcf* genes except *tcf7l2* are expressed in the posterior hypothalamus. T, telencephalon; Ha, habenula; TeO, tectum opticum; DT, dorsal thalamus (thalamus); Hy, hypothalamus. Scale bars: 100 μm.

Tcf expression and canonical Wnt signaling activity persist in the adult hypothalamus

To determine whether Wnt-responsive cells exist in the hypothalamus at later stages, we examined brains from larvae at 10 days postfertilization and 6-month-old adults. At larval stages, specific expression of *tcf7l1a/b* and *tcf7l2* was not observed in the posterior hypothalamus (data not shown). By contrast, *lef1*, *tcf7*, and *top:dgfp* were all expressed in the caudal

zone of the periventricular hypothalamus (Fig. 2A–C). The expression pattern of *top:dgfp* appeared to overlap with the combined domains of *lef1* and *tcf7*, with higher intensity in the zone closest to the posterior recess of the diencephalic ventricle (Fig. 2D–F). We found that the expression of *lef1*, *tcf7*, and *top:dgfp* also persists in the adult brain, specifically in the caudal zone of the periventricular hypothalamus (Fig. 2G–I). Cross sections through this region of the adult brain indicated that *lef1*, *top:dgfp*, and *axin2* (a candidate Wnt target[95]) are strongly expressed in the posterior recess of the diencephalic ventricle (Fig. 2J–L), where potential adult stem cells reside as marked by labeling with PCNA and BrdU.[86] These data suggest that canonical Wnt signaling may be involved in adult hypothalamic neurogenesis.

Characterization of Wnt-responsive and Lef1-positive cell types within the embryonic hypothalamus

To further investigate the potential role of canonical Wnt signaling in hypothalamic neurogenesis, we determined which cell types expressed TOP:dGFP and Lef1 protein in 50 hpf embryos. We found that in the posterior hypothalamus, TOP:dGFP spanned the proximal to distal extent of this region (Fig. 3A), in which we observed a gradient of neuronal differentiation. The neuronal marker HuC/D was primarily restricted to cells more proximal to the thalamus (Fig. 3B), while the proliferation marker PCNA was mainly expressed in distal cells (Fig. 3C). Cross-section analysis revealed that TOP:dGFP-positive cells were either negative or weakly positive for HuC/D (circle, Fig. 3D). Some of these cells also co-expressed PCNA (circle, Fig. 3E), suggesting that the Wnt-responsive population spans the period of cell cycle exit and early differentiation. We found that some TOP:dGFP-positive cells expressed serotonin (5-HT, circle, Fig. 3F), but did not express TH, GABA, or the glial marker GFAP (Fig. 3G–I). These data indicate that Wnt-responsive cells may adopt particular neuronal fates characteristic of their position in the hypothalamus. Taken together, TOP:dGFP-positive cells in the 50 hpf hypothalamus are likely to be neural progenitors, precursors, and specific subtypes of neurons.

Surprisingly, we found that Lef1 and TOP:dGFP expression overlapped only occasionally (circle, Fig. 4A). Similar to the Wnt-responsive cells described above, some Lef1-positive cells were weakly positive for HuC/D and others were

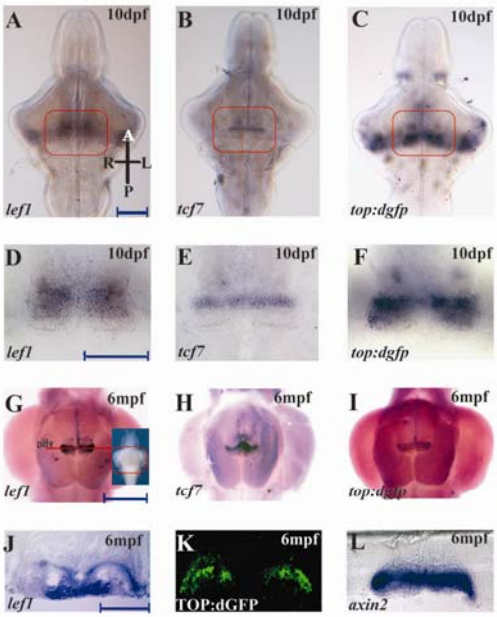

FIG. 2. Expression of *tcf* genes and *top:dgfp* in the larval and adult zebrafish hypothalamus. (**A–C**) Ventral view of 10 dpf larval brains; orientation is indicated in panel (**A**). (**D–F**) Higher power images of the fields indicated in (**A–C**). *top:dgfp* Expression encompasses the combined domains of *lef1* and *tcf7*. (**G–I**) Ventral views of 6mpf adult brains. (**J**) Cross section through adult hypothalamus at the level indicated in panel (**G**). (**K**) GFP antibody staining on an adult TOP:dGFP hypothalamus cross section at the same level as panel (**J**). (**L**) *axin2* expression on an adult hypothalamus cross section at the same level as panel (**J**). *lef1* and GFP/*axin2* occupy different regions of the periventricular zone. pHy, periventricular hypothalamus. Scale bars: (**A, D**) 100 μm; (**G**) 500 μm; (**J**) 200 μm.

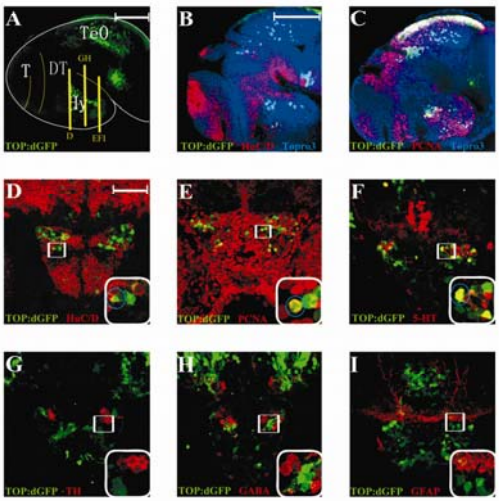

FIG. 3. Immunohistochemical identification of Wnt-responsive cells in the 50 hpf embryonic hypothalamus. (**A**) Lateral view of TOP:dGFP whole-mount brain stained with GFP antibody, observed in a 100-μm confocal projection. (**B, C**) Triple labeling for TOP:dGFP, HuC/D or PCNA, and Topro3, observed in a single confocal slice. (**D–I**) Twelve-micron cryosections immunostained for the markers listed in each panel. Positions of cross sections are indicated in panel (**A**). Boxed region is shown at higher magnification in lower right corner, and circled cells are double labeled. GFP staining partially overlaps with HuC/D, PCNA, and 5-HT, but not with TH, GABA, or GFAP. T, telencephalon; TeO, tectum opticum; DT, dorsal thalamus (thalamus); Hy, hypothalamus. Scale bars: (**A, B**) 100 μm; (**D**) 50 μm.

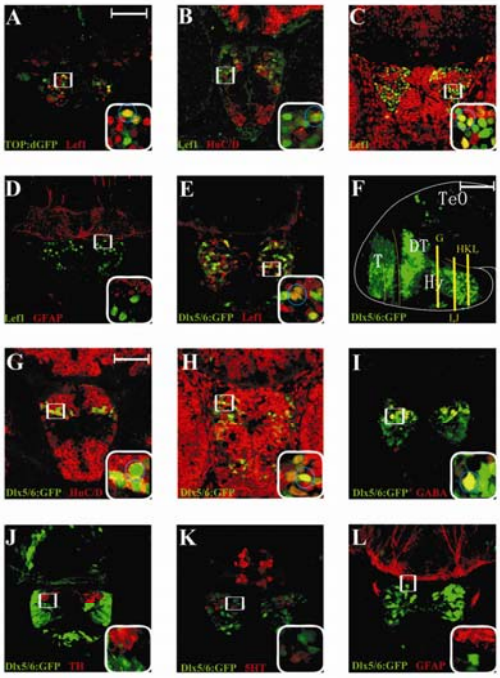

FIG. 4. Immunohistochemical identification of Lef1-expressing cells in the 50 hpf embryonic hypothalamus. (**A–E**) Twelve-micron cryosections immunostained for the markers listed in each panel. Positions of cross sections for panels (**A**) and (**C–E**) are at the same level as Figure 3E, and panel (**B**) is at the same level as Figure 3G. Boxed region is shown at higher magnification in lower right corner, and circled cells are double labeled. Lef1 staining partially overlaps with TOP:dGFP, HuC/D, PCNA, and *dlx5/6:gfp*, but not with GFAP. (**F**) Lateral view of *dlx5/6:gfp* whole-mount brain, observed with a 100-μm confocal projection. (**G–L**) Twelve-micron cryosections immunostained for the markers listed in each panel. Positions of cross sections are indicated in panel (**F**). Boxed region is shown at higher magnification in lower right corner, and circled cells are double labeled. GFP staining partially overlaps with HuC/D, PCNA, and GABA, but not with TH, 5-HT, or GFAP. T, telencephalon; TeO, tectum opticum; DT, dorsal thalamus (thalamus); Hy, hypothalamus. Scale bars: (**A, G**) 50 μm; (**F**) 100 μm.

positive for PCNA (circles, Fig. 4B, C). In addition, none of the Lef1-positive cells expressed GFAP (Fig. 4D). Due to difficulties with antibody specificity, we were unable to directly analyze the neuronal subtypes of Lef1-positive cells. However, we took advantage of a transgenic fish line that expresses GFP driven by a *dlx5/6* enhancer.[92] These two genes have been reported to label GABAergic progenitors and precursors in mouse.[96,97] We found that many of the *dlx5/6:gfp*-positive cells in the posterior hypothalamus co-expressed Lef1 (circle, Fig. 4E). The transgene labels a large number of cells within the posterior hypothalamus, spanning both post-mitotic and progenitor regions (Fig. 4F–H, circled cells are double labeled). Immunohistochemical analysis revealed that as in mouse, GFP-positive cells were primarily GABAergic (circle, Fig. 4I), and we did not detect overlap with TH, 5-HT, or GFAP (Fig. 4J–L). This indirect analysis suggests that Lef1-positive cells in the 50 hpf hypothalamus are likely to be neural progenitors, GABAergic precursors, and immature neurons.

Characterization of Wnt-responsive cells within the adult hypothalamus

We found that cells with strongest expression of TOP:dGFP in the adult hypothalamus are mainly located adjacent to the diencephalic ventricle (Fig. 5A). We further analyzed these cells in cross sections through the diencephalic ventricle, and found that TOP:dGFP-positive cells did not express HuC/D, PCNA, or GFAP, but instead were located between the HuC/D and PCNA/GFAP-positive cell populations (Fig. 5B–D). Because HuC/D labels postmitotic neurons, and PCNA labels proliferating cells, while GFAP has also been reported to be expressed in adult astrocyte-like neural stem cells,[98] our data suggest that Wnt-responsive cells in the adult hypothalamus occupy a developmental state between proliferating progenitors/stem cells and postmitotic neurons.

Discussion

Wnt-responsive cells in the hypothalamus are likely to be neuronal precursors

Based on our data, Wnt-responsive and Lef1-expressing cells in the 50 hpf embryonic hypothalamus are most likely to be a subset of mitotic progenitors and postmitotic precursors,

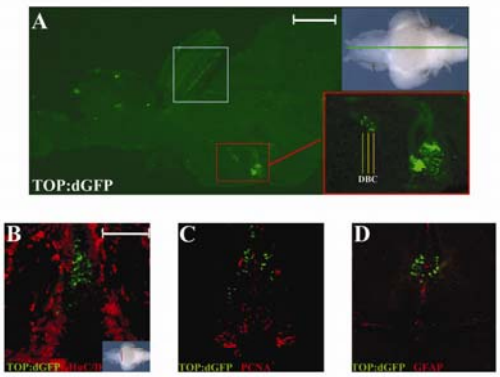

FIG. 5. Immunohistochemical identification of Wnt-responsive and Lef1-expressing cells in the adult hypothalamus. (**A**) GFP antibody staining on a sagittal section through the midline of a TOP:dGFP adult brain. Specific expression of GFP is observed in the optic tectum (white box) and the periventricular hypothalamus (red box). (**B–D**) Twenty-five-micron hypothalamic cross sections immunostained for the markers listed in each panel. GFP-positive cells do not express HuC/D, PCNA, or GFAP. Scale bars: (**A**) 300 μm; (**B**) 100 μm.

although Wnt reporter expression may persist as cells become immature neurons due to the rapid rate of differentiation. Wnt-responsive cells in the adult are most likely to be post-mitotic precursors, residing between mitotic progenitors and neurons. It is also possible that these cells could be quiescent (PCNA-negative) neural stem cells, as it is known that the cell cycle inhibitor p21 promotes degradation of PCNA during an extended G1 phase.[99] However, their position and lack of GFAP expression makes this possibility unlikely. A core question remains as to what role the canonical Wnt signaling pathway plays in the process of precursor maintenance and specification.

Our data also suggest that the regulation of canonical Wnt signaling inside the hypothalamus may be modified by cell-intrinsic states. The expression patterns of TOP:dGFP and Lef1 are not fully overlapping, and in the posterior hypothalamus, where Lef1 is strongly expressed at 50 hpf, the intensity of TOP:dGFP is not particularly strong. This suggests that other Tcfs such as *Tcf7* and *Tcf7l1a/b*, which are expressed in this region as well, may compete to keep canonical Wnt activity at an intermediate level. Further, GFP-positive cells express 5-HT, while Lef1-positive cells appear to become GABAergic, indicating that these represent two divergent lineages. Cells that have stopped responding to Wnt signals may retain Lef1 protein for some time, as it is known that Lef1 expression itself is upregulated by the Wnt pathway through autoregulation.[54,100] In addition, alternative *lef1* transcripts encoding dominant-repressor forms of the protein have been reported in other contexts, and these isoforms could be recognized by our antibody and mRNA probe. Further, other Wnt pathway modulators could also influence the transcriptional output.

Wnt signaling and neural stem cells

Canonical Wnt signaling has been suggested to be generally responsible for the expansion of neural stem cells or progenitor pools in previous publications. However, in the adult brain we found strong TOP:dGFP expression only in the optic tectum and hypothalamus, while at least 10 distinct regions within the adult zebrafish brain have been identified as proliferation zones with neural progenitors.[86,101] It is therefore unlikely that the canonical Wnt signaling pathway is a generally required factor for all neural stem cell maintenance. Instead, the more restricted expression pattern we observe suggests that Wnt-responsive cells may be limited to particular progenitor or precursor populations, and those lineages may be as evolutionarily conserved as Wnt signaling itself. It has been suggested that postnatal neural stem cells are likely to be fate restricted,[102,103] which further supports the idea that Wnt signaling is required for specific neurogenesis processes.

Intriguingly, Tcf7l1 has been reported to function as a transcriptional repressor in the maintenance of ESCs,[77] which may suggest that inhibition of Wnt transcriptional targets is a key condition for stem cell/progenitor maintenance. As most previous studies have focused on the ultimate phenotypes following Wnt pathway manipulation in the brain, it is not clear whether particular transcriptional targets are repressed or activated in these instances. It is therefore important to understand the mechanism of Tcf function in detail when examining the more general role of Wnt signaling in vertebrate neurogenesis.

Adult neurogenesis and the hypothalamus

Adult neurogenesis has become an attractive target for potential therapeutic strategies in neural degenerative diseases and injury, and the Wnt signaling pathway has also been suggested to have therapeutic potential for Alzheimer's disease and Parkinson's disease.[35,104] The generation of new neurons in the adult mammalian brain has long been thought to be restricted primarily to two regions: the subventricular zone of the lateral ventricle, which generates olfactory bulb GABAergic interneurons via the rostral migratory stream, and the subgranular zone of the dentate gyrus, which generates hippocampal granular neurons, both in the telencephalon. Recently, investigation has revealed the diencephalic third ventricle as another proliferating region in mammalian forebrain, contributing to the adult hypothalamic neurogenesis.[105,106] The hypothalamus has also been confirmed as a site of adult neurogenesis in the teleost,[86,87,107] highlighting this part of the brain as a good model to investigate adult neurogenesis in both mammalian and nonmammalian vertebrates.

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Disclosure Statement

No competing financial interests exist.

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CHAPTER 3

AN EVOLUTIONARILY CONSERVED ROLE FOR WNT SIGNALING IN HYPOTHALAMIC PROGENITOR DIFFERENTIATION

Abstract

Previous studies have raised the possibility that Wnt signaling may regulate both neural progenitor maintenance and neuronal differentiation within a single population. Here we investigated the role of Wnt/ß-catenin activity in the zebrafish hypothalamus and found that the pathway is first required for the proliferation of unspecified hypothalamic progenitors in the embryo. At later stages, including adulthood, Wnt activity is required transiently for the differentiation of neural progenitors and negatively regulates radial glia differentiation. The presence of Wnt activity is conserved in hypothalamic progenitors of the adult mouse, where it plays a conserved role in inhibiting the differentiation of radial glia. This study establishes the vertebrate hypothalamus as a model for Wnt-regulated postembryonic neural progenitor differentiation, and demonstrates that the general role of Wnt signaling in this tissue is evolutionarily conserved.

Introduction

While neurogenesis was originally defined as a phenomenon exclusive to developing brains, it has now been identified in the adult CNS of mammals [1] and non-mammalian vertebrates.[1, 2] The regulation of postembryonic neurogenesis is thus a critical modulator of CNS homeostasis and plasticity, and the molecular mechanisms underlying this process are of obvious intense interest. One of the best-characterized signaling pathways involved in developmental neurogenesis is the regulation of target gene transcription by

Wnt/ß-catenin signaling. For example, the hippocampus of null mutants for *Wnt3a*, *LRP6* and *Lef1*, contains a smaller dentate gyrus, with reduced production of granule neurons and abnormalities in radial glial scaffolding due to proliferation and patterning defects.[3-5] These results suggest that Wnt activity may be required for the proliferation and normal differentiation of embryonic neural progenitors at early stages.

Recently, several studies with conditional approaches have revealed new aspects of Wnt signaling in postembryonic neurogenesis. One prevailing model suggests that Wnt activity is required to keep neural progenitors undifferentiated.[6, 7] However, other data indicate that Wnt activity can both promote and inhibit neuronal differentiation.[8] Consistent with these conflicting outcomes, hippocampal progenitors persist as $GFAP^{+}$ radial stem-like cells when Wnt function is lost, while activation of the pathway in the rostral migratory stream, by *Apc* deletion, results in developmental arrest of $Ascl1^{+}$ transit amplifying cells.[9, 10] Together, all these studies suggest an untested unifying model in which Wnt activity is transiently required for an early step of the neurogenesis pathway.

We have previously shown that Wnt signaling through Lef1 is required for neurogenesis in the embryonic zebrafish hypothalamus.[11] Subsequently, we found that both Wnt signaling and neurogenesis continue in the zebrafish hypothalamus through adult stages.[12] Compared with other forebrain regions, the hypothalamus is relatively unstudied as a model of postembryonic neurogenesis. While the hypothalamus has been identified as a region with proliferation and neurogenesis in adult mammals, the regulation and function of this regional activity is poorly understood.[13-15] Hypothalamic

neurogenesis could be significant in the regulation of multiple autonomic and endocrine pathways, as already demonstrated with feeding behavior.[16] The presence and gene expression profile of specific neuronal lineages, such as Dlx^+ GABAergic precursors, is similar to that of other brain regions.[17] In addition, the hypothalamus contains persistent radial glial tanycytes that are essential for the endocrine function and may also serve as a neural progenitor population.[18] However the regulation of tanycyte differentiation and maintenance has remained uncharacterized.

Here we identify Wnt-responsive cells in developing and postembryonic zebrafish hypothalamus, and find evidence for reiterated pathway activation first in unspecified progenitors, and again later in postmitotic neural progenitors. Consistent with the profile of pathway activity, we find that Wnt signaling is first required for proliferative expansion of unspecified progenitors in the embryo and later regulates the differentiation of GABAergic and serotonergic lineages as well as radial glia. Significantly, our data indicate that Wnt signaling must be activated transiently in order for neuronal differentiation to occur. Finally, we show that Wnt activity is present in ventricular and parenchymal progenitors of the adult mouse hypothalamus. In this system, we find that although *Hes1*$^+$ ventricular progenitors do not produce neurons, the role of Wnt signaling in radial glial development and maintenance is evolutionarily conserved. Together, these data lead to a general model for Wnt function in postembryonic neural progenitor differentiation.

Results

Wnt pathway components are continuously expressed in the posterior ventricular zebrafish hypothalamus

We previously showed that Wnt signaling and the transcriptional mediator Lef1 regulate embryonic hypothalamic neurogenesis.[11] In subsequent work, we found that Wnt pathway activity was present in the hypothalamus throughout the life of the animal.[12] To understand the role of the Wnt pathway in the postembryonic hypothalamus, we first focused on the specific expression of the three strongest candidates for modulating Wnt/ß-catenin activity, *lef1*, *tcf7*, and *wnt8b,* at three different stages.

At 32 hours postfertilization (hpf), *wnt8b* is expressed along the 3rd ventricle, most strongly in the posterior region including the presumptive posterior recess. *lef1* is expressed adjacent to the *wnt8b*-expressing domain in the posterior region, with additional expression in more anterior regions. Expression of a *tcf7:GFP* reporter transgene overlaps with *lef1*, but is stronger in more anterior regions and the medial posterior region (Supplementary Fig. 3.1A).[19]

By 4 days postfertilization (dpf), the 3rd ventricle and its associated posterior recess serve as reliable hypothalamic landmarks. At this stage *wnt8b* expression persists in the posterior 3rd ventricle and the elongated posterior recess, forming an inverted "T" shape. *lef1* is broadly expressed in the posterior hypothalamic region, adjacent to the *wnt8b*-expressing domain, while *tcf7* reporter expression remains overlapping with *lef1*, and is stronger in the rostral zone of the posterior region (Supplementary Fig. 3.1B).

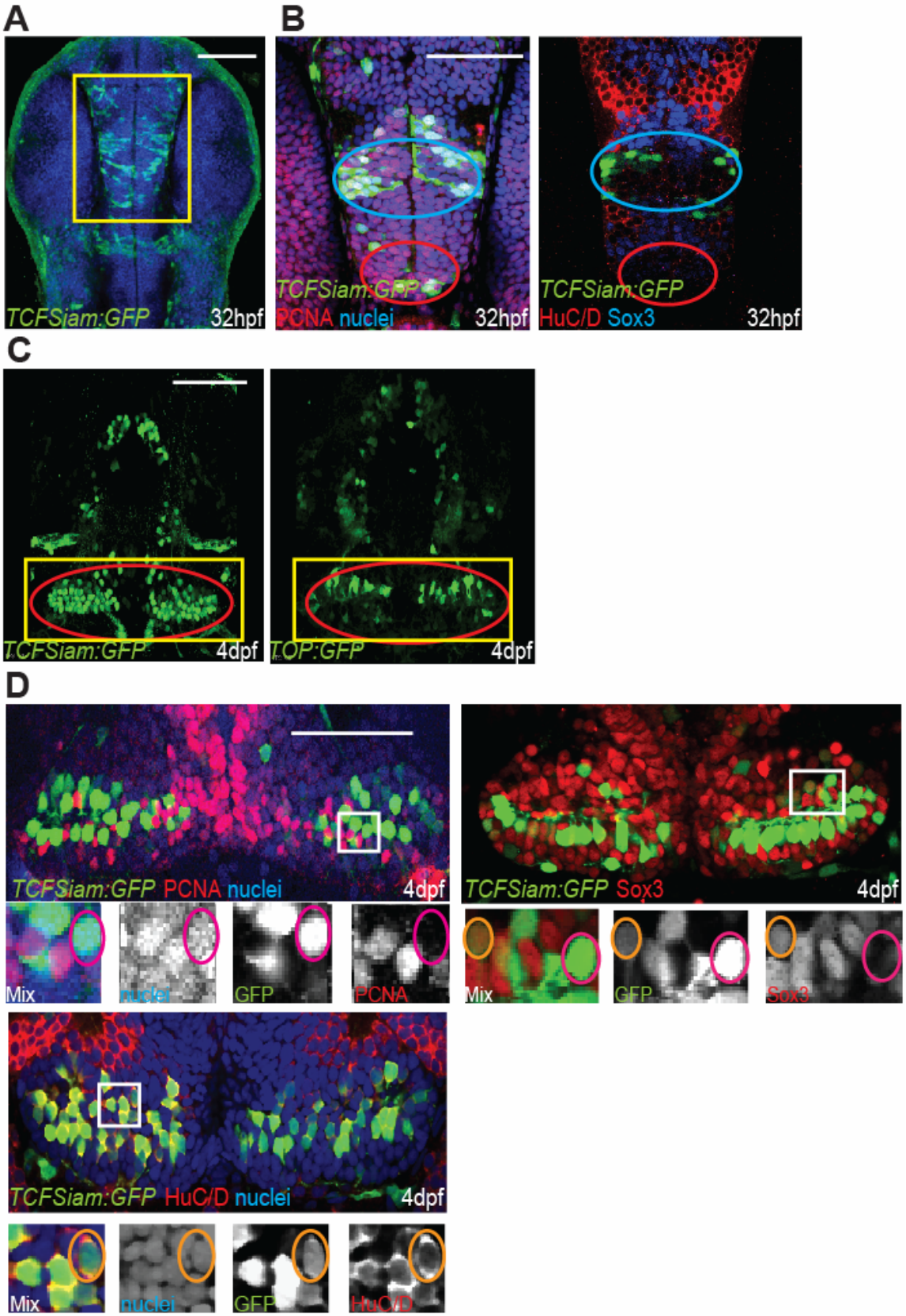
In whole-mount views of the adult hypothalamus, *tcf7* reporter expression suggests a similar pattern as seen at 4dpf (Supplementary Fig. 3.1C). To analyze deeper structures, we used sagittal slices and examined *lef1* and *tcf7* reporter expression adjacent to the 3rd ventricle and the posterior recess, where *wnt8b* is expressed, consistent with our findings at 4dpf (Supplementary Fig. 3.1D). These data are consistent with a model of continuous Wnt activity associated with the ventricular region of the posterior hypothalamus.

Wnt activity is present in unspecified hypothalamic progenitors
at early embryonic stages

Because multiple *tcf* genes are expressed in the hypothalamus at all stages, we chose to characterize Wnt-responsive cells using transcriptional reporter lines. We used both *TOP:GFP*, a destabilized Wnt reporter as well as *TCFSiam:GFP*, a stable Wnt reporter, to distinguish between Wnt-responsive cells and their progeny.[20, 21] To confirm the difference in reporter responsiveness, we used the *hs:dkk1* transgene to conditionally inhibit canonical Wnt signaling in both reporter lines and monitored the decrease in GFP expression following heat shock.[22] We found that it took 24 hours to achieve an 80% reduction in *TCFSiam:GFP*$^{+}$ cells, compared to only 8 hours for *TOP:GFP* (Supplementary Fig. 3.2).

At 32hpf, we found that Wnt-responsive cells were present in the presumptive lateral and posterior recess regions (Fig. 3.1A). At this embryonic stage, almost all *TCFSiam:GFP*$^{+}$ and *TOP:GFP*$^{+}$ cells were PCNA^{+} (Fig. 3.1B, Table 3.1). In contrast,

Figure 3.1 Identification of Wnt-responsive cells in the zebrafish hypothalamus. (A) Ventral view of *TCFSiam:GFP.* Yellow box marks the area shown in (B). (B) Optical sections of co-staining with PCNA, Sox3, and Hu. Blue ovals label the presumptive lateral recess, and red ovals label the presumptive posterior recess. (C) Ventral Z-projections of 4dpf *TCFSiam:GFP* and *TOP:GFP*. Yellow box marks the area shown in (D, E), and red ovals label the posterior recess. (D) Co-staining of *TCFSiam:GFP* with PCNA, Sox3, and HuC/D. (E) Co-staining of *TCFSiam:GFP* with *dlx5/6:gfp* and serotonin. (F) Ventral and sagittal views of *TCFSiam:GFP* in the adult hypothalamus. Yellow box marks the area shown in (G), and red oval labels the presumptive posterior recess. (G) Co-staining of *TCFSiam:GFP* with Sox3, Hu, 5HT, *dlx5/6:mCherry*, and GABA in the adult posterior hypothalamus. Small orange circles label cells with colocalization, and small magenta circles label cells without colocalization. Scale bars: (A-E, G) 80µm, (F) 250µm.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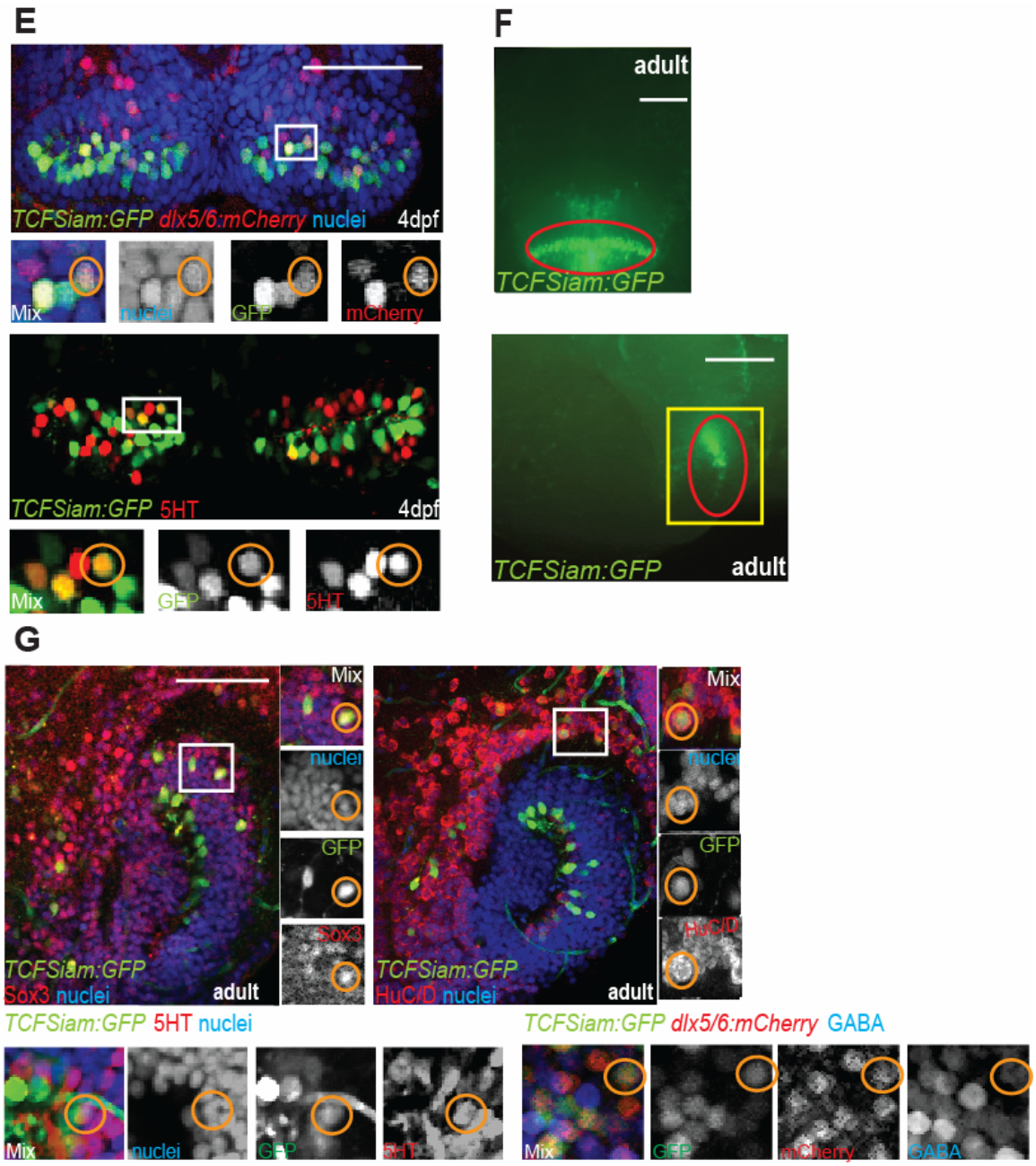


(Figure3.1 continued)

Table 3.1 Coexpression percentage of cell type markers with reporters. Cells were counted from at least three individual samples for each set of markers. Error=±SD.

	PCNA	**Sox3**	**HuC/D**	***dlx5/6:mCherry***	**GABA**	**5HT**
32hpf						
TOP:GFP	97.3±1.5	13.1±3.5	1.3±0.5	-	-	-
TCFsiam:GFP	96.2±1.2	7.2±2.3	2.1±0.8	-	-	-
4dpf						
TOP:GFP	5.2±1.3	89.2±7.1	5.2±2.1	7.1±3.2	1.7±1.1	2.9±0.5
TCFsiam:GFP	9.3±1.7	40.3±9.9	78.2±9.3	48.1±9.9	31.2±5.0	17.2±4.3
tcf7:GFP	4.3±2.3	68.1±11.7	81.2±6.8	26.1±7.2	16.1±4.1	30.1±3.2
dlx5/6:GFP	9.2±1.2	39.2±7.1	61.3±5.4	-	73.1±6.1	0
Adult						
TOP:GFP	<1	98.1±1.1	3.1±2.4	<1	<1	1.1±0.7
TCFsiam:GFP	<1	47.3±3.4	66.3±5.3	37.0±4.9	7.1±1.8	5.2±1.5
tcf7:GFP	<1	59.1±4.2	73.1±4.1	-	9.1±1.6	7.3±2.5

few *TCFSiam:GFP*$^{+}$ and *TOP:GFP*$^{+}$ cells were Sox3^{+}, mostly in the presumptive lateral recess region (Blue oval in Fig. 3.1B, Table 3.1), and co-localization of both reporters with the neuronal marker HuC/D was very low (Fig. 3.1B, Table 3.1). These data suggest that Wnt-responsive cells in the 32hpf zebrafish hypothalamus are proliferating unspecified progenitors, some of which may be in transition to Sox3^{+} neural progenitors.

Wnt signaling is active in hypothalamic neural progenitors
at postembryonic stages

At 4dpf, ventral confocal Z-projections of *TCFSiam:GFP* and *TOP:GFP* suggested that most Wnt-responsive cells are located in the posterior recess (Red oval in Fig. 3.1C). We focused on the posterior recess for further analysis, because it had a higher density of reporter-expressing cells. At this stage, we found that Wnt-responsive cells were primarily PCNA^{-}/Sox3^{+} neural progenitors, although some were PCNA^{+}/Sox3^{+}, indicating a transition through cell cycle exit (Fig. 3.1D, Supplementary Fig. 3.4, and Table 3.1). BrdU labeling also suggested that the majority of 4dpf Wnt-responsive cells were not proliferating (Supplementary Fig. 3.3).

At 4dpf, we also used GFP perdurance in reporter lines to determine which lineages derive from the Wnt-responsive cells. Both Wnt reporters were coexpressed with the pan-neuronal marker HuC/D, the GABAergic lineage markers *dlx5/6:mCherry* and GABA, and serotonin. However, coexpression was relatively low in cells expressing the destabilized reporter *TOP:GFP* (Supplementary Fig. 3.4, Table 3.1) and much higher in

cells expressing the stable reporters *TCFSiam:GFP* and *tcf7:GFP* (Fig. 3.1D,E, Supplementary Fig. 3.4, Table 3.1). Because we did not observe any overlap between markers for the GABAergic and serotonergic lineages (Supplementary Fig. 3.5), these experiments suggest that Wnt-responsive cells generate at least two different neuronal lineages. Furthermore, the different compositions of the destabilized and stable GFP reporter populations suggest that active Wnt signaling is reduced as neuronal differentiation proceeds.

In the adult hypothalamus, both ventral and sagittal views of *TCFSiam:GFP* and *TOP:GFP* confirmed a similar distribution of Wnt-responsive cells in the posterior recess region (Red ovals in Fig. 3.1F). We previously reported that *TOP:GFP*$^{+}$ cells in the adult hypothalamic lateral recess did not express the proliferation marker PCNA, the postmitotic neuronal marker HuC/D, or GFAP.[12] To determine the identity of GFP^{+} cells in the posterior recess, we performed whole-mount sagittal analysis of the posterior recess using 150μm confocal projections taken from the midline. We found that Sox3 continues to be expressed in the majority of Wnt-responsive cells in the posterior recess (Fig. 3.1G, Supplementary Fig. 3.4, and Table 3.1). The expression of neuronal markers still distinguished the destabilized and stable reporters, as many *TCFSiam:GFP*$^{+}$ and *tcf7:GFP*$^{+}$ cells, but very few *TOP:GFP*$^{+}$ cells, co-expressed HuC/D (Fig. 3.1G, Supplementary Fig. 3.4 and Table 3.1). In addition, we found that adult hypothalamic Wnt-responsive cells continue to contribute to the GABAergic and serotonergic lineages identified in 4dpf hypothalamus (Fig. 3.1G, Supplementary Fig. 3.4, and Table 3.1).

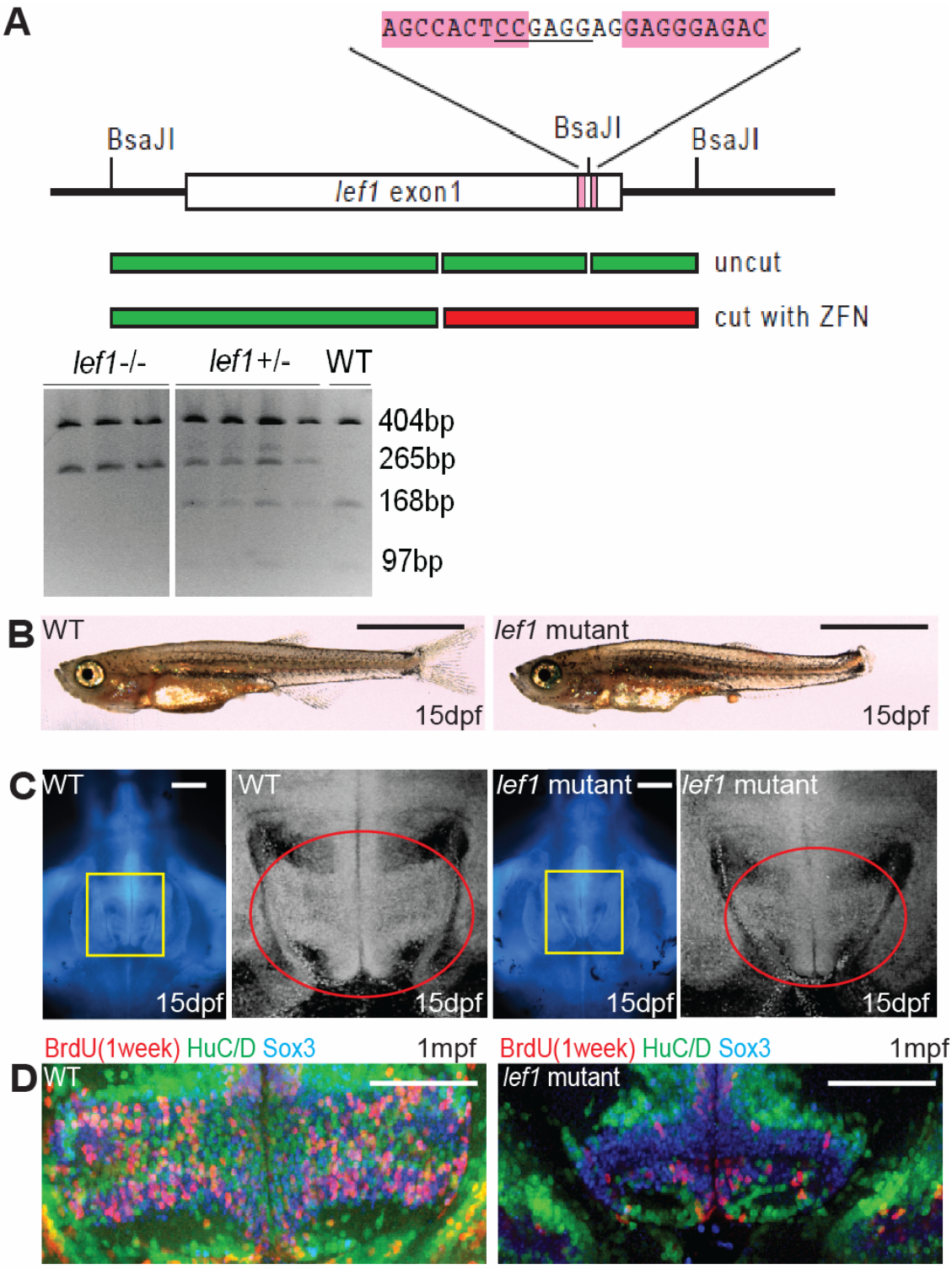
Together, these analyses suggest that in the posterior recess region of the 4dpf and adult zebrafish hypothalamus, Wnt activity is transiently present in $Sox3^{+}$ GABAergic and serotonergic progenitors as they become postmitotic.

lef1 is required for postembryonic hypothalamic neurogenesis

Mutations in individual *Tcf* genes often have only minor defects, allowing us to investigate the role of Wnt activity in neurogenesis in the context of grossly normal morphology.[19, 23] Among the five *tcf* genes in zebrafish, *lef1* was the most promising candidate to regulate hypothalamic neurogenesis. Mouse *Lef1* mutants exhibit a smaller dentate gyrus with neurogenesis defects in the hippocampus, but hypothalamic phenotypes have not been explored.[4, 5] In zebrafish *lef1* morpholino knockdown produces neurogenesis defects in the hypothalamic GABAergic lineage, and we found that Lef1 is co-expressed with *dlx5/6:GFP* at 4dpf (Supplementary Fig. 3.5).[11]

To explore the later functions of *lef1* in the hypothalamus, we generated a *lef1* mutant using zinc finger nuclease-mediated gene targeting.[24] Genomic (Fig. 3.2A) and immunohistochemical (not shown) analyses indicated that this allele is a functional null, and we observed similar phenotypes to two other *lef1* null alleles generated by ENU mutagenesis.[21, 25] At 15dpf, *lef1* mutants can be identified by their smaller fins. While mutant fish exhibit similar body mass and brain size as their wild-type siblings (Fig. 3.2B,C), we observed a significant decrease in the size of the posterior hypothalamus. Using confocal volume reconstruction, we found the mutant posterior hypothalamus is

Figure 3.2 *lef1* is required for proliferation and neurogenesis in the postembryonic hypothalamus. (A) *lef1* ZFN target region and genotyping. (B) Whole fish and (C) brain size comparisons of *lef1* mutant to wild-type at 15dpf. Boxed region in left panel is enlarged on right, posterior recess is circled. (D) Expression of HuC/D, Sox3, and 7-day BrdU labeling in posterior recess of wild-type and *lef1* mutant hypothalamus. (E) Quantification of posterior hypothalamus size. (F) Tracing of proliferating cells. *lef1* mutants have a smaller $Sox3^{+}$ progenitor pool, but a higher percentage of $BrdU^{+}$ cells express Sox3 and fewer produce HuC/D^{+} ,$GABA^{+}$, or $5HT^{+}$ neurons. All cell counts were collected from three individual samples for each genotype and calculated using Volocity software, and brain volumes were calculated using Amira software. Scale bars: (B) 2mm. (C) 200µm. (D) 100µm. *: $p<0.05$, **: $p<0.005$.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265bp
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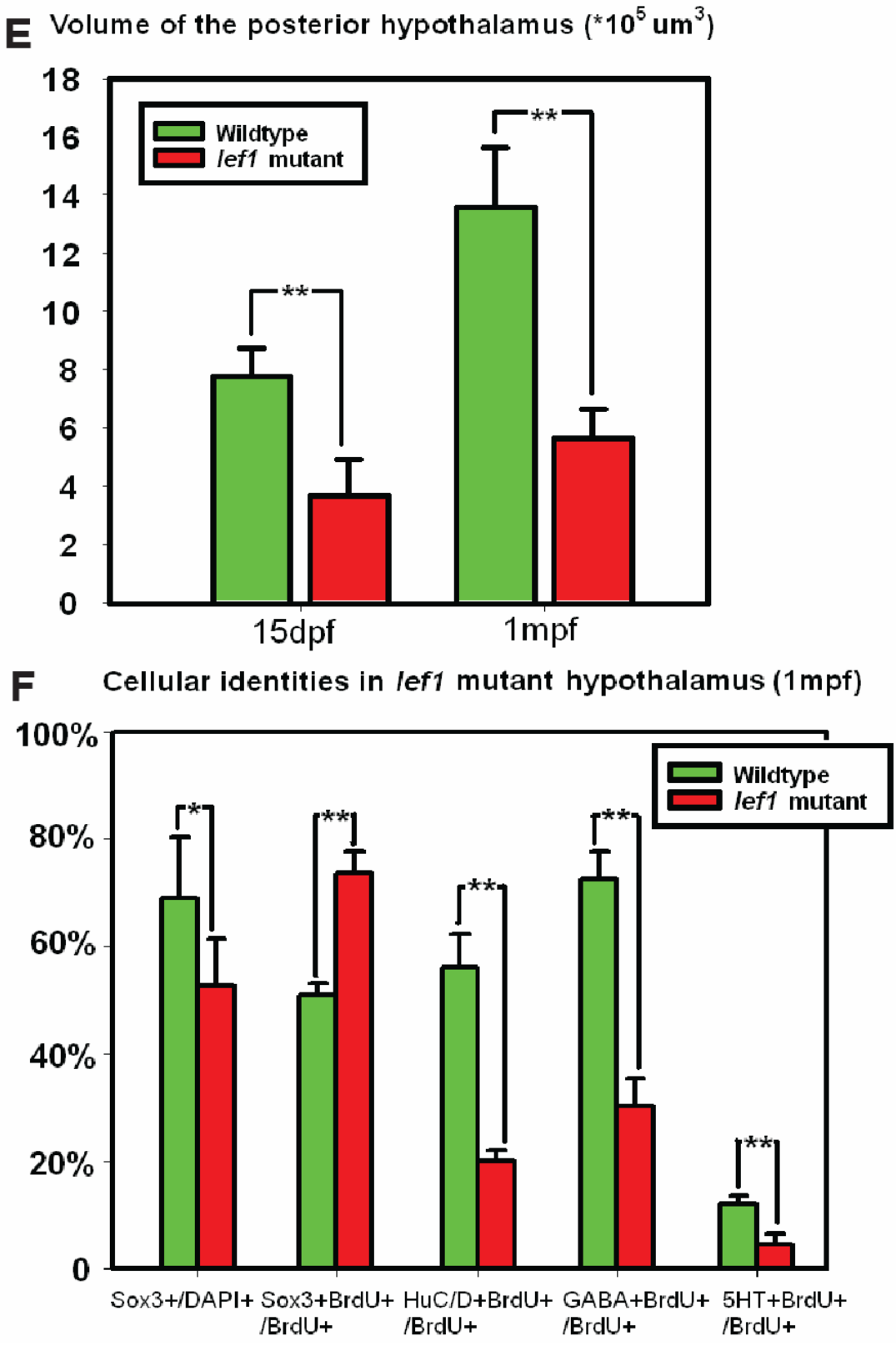


(Figure3.2 continued)

50% of wild-type size (Fig. 3.2C,E), suggesting a specific proliferation defect in this tissue.

At 1 month postfertilization (mpf), the *lef1* mutant posterior hypothalamus is only 40% of wild-type size (Fig. 3.2E), and 1 week BrdU tracing showed few proliferating cells in this region, with most of the remaining $BrdU^+$ cells located near the midline (Fig. 3.2D). In addition, the HuC/D^+ neuronal population in the paraventricular posterior recess region is almost completely absent in *lef1* mutants (Fig. 3.2D). While the $Sox3^+$ neural progenitor pool is also smaller, BrdU labeling suggested that the few proliferating cells are arrested in the $Sox3^+$ state and unable to progress into HuC/D^+ neurons of either the GABAergic or serotonergic lineages (Fig. 3.2D,F).

While our previous data suggested a more significant role for *lef1* in early hypothalamic development, morpholino-based studies can be subject to off-target effects or targeting of maternally deposited pre-mRNA. Our current analysis of zygotic mutants suggests that at postembryonic stages through adulthood, cells in the *lef1* mutant hypothalamus fail to proliferate or differentiate normally and are arrested as $Sox3^+$ neural progenitors.

Transient Wnt signaling is required for hypothalamic neurogenesis throughout life

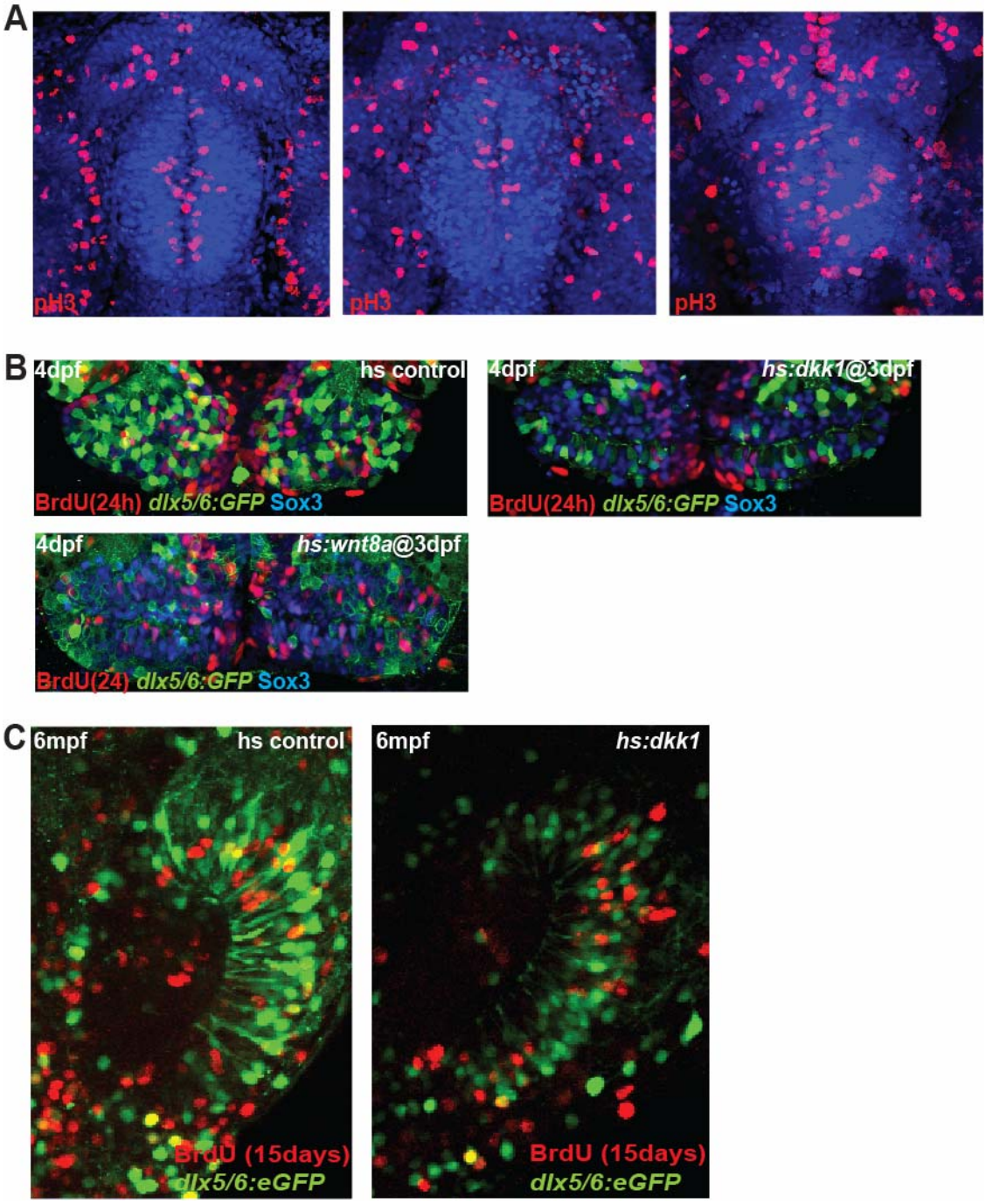
The phenotype of *lef1* mutants suggested a continuous requirement for Wnt signaling in hypothalamic neurogenesis, but the lack of conditional mutagenesis approaches in

zebrafish precluded us from fully testing this hypothesis. We therefore used a series of transgenic lines to perform conditional gain- and loss-of-function assays at early embryonic (24-32hpf), later embryonic (3-4dpf), and adult (6mpf) stages. We used four different heat-shock lines to manipulate Wnt/ß-catenin signaling: *hs:wnt8a* to activate the pathway, and *hs:dkk1*, *hs:axin1*, and *hs:*Δ*tcf* to inhibit the pathway.[22, 26, 27]

Because our data suggested a role for Wnt signaling in unspecified proliferating progenitors at 32hpf, we first analyzed the effects on proliferation following heat shock. A 2-hour BrdU pulse was performed at 22hpf, followed by heat shock at 24hpf, and fixation at 32hpf. We found that the number of $pH3^+$ cells in the hypothalamic region was significantly increased following Wnt activation and decreased following Wnt inhibition (Fig. 3.3A,D), suggesting that Wnt signaling regulates overall proliferation levels. In addition, we found similar effects on the number of $BrdU^+/Sox3^+$ neural progenitors (Fig. 3.3D), suggesting canonical Wnt signaling is also necessary and sufficient for the expansion of this population. These data are consistent with existing models of Wnt as a mitogen in progenitor cells.[28]

Our analysis of Wnt activity at 4dpf suggested a potential role for the pathway in neuronal differentiation. We therefore analyzed the effects on proliferation and differentiation following heat shock at this stage. Because the number of $pH3^+$ cells in the posterior recess at 4dpf is very low, we used BrdU exclusively to measure proliferation. A 2-hour BrdU pulse was performed at 70hpf, followed by multiple heat shocks every 8 hours, and fixation at 96hpf. In control embryos, we found that the

Figure 3.3 Wnt signaling has different roles in hypothalamic neurogenesis at different stages. (A) Phospho-Histone H3 staining in the 32hpf hypothalamus. (B) Co-localization of 24-hour BrdU labeling with Sox3 and *dlx5/6:GFP* labeling in the 4dpf posterior hypothalamus. (C) Co-localization of 15-day BrdU labeling with *dlx5/6:GFP* in the adult hypothalamus. (D) $pH3^+$ and $BrdU^+/Sox3^+$ cell numbers in the 32hpf hypothalamus following 2-hour labeling and Wnt pathway inhibition or activation at 24hpf. (E) $BrdU^+$ cell numbers in the 4dpf hypothalamus following 2-hour labeling and Wnt pathway inhibition or activation at 3dpf. (F) $BrdU^+$ cell fates in the 4dpf hypothalamus following labeling and Wnt pathway inhibition or activation at 3dpf. (G) $BrdU^+$ cell fates in the adult hypothalamus following 2-day labeling and Wnt pathway inhibition or activation for 15 days. (H) mRNA expression in the dissected hypothalamus following Wnt pathway inhibition or activation for 15 days. All cell counts were gathered from five samples for each genotype and processed with Volocity software. Each template for quantitative RT-PCR was generated from three dissected adult hypothalami. Scale bars: (A, B) 50μm, (C, D) 80μm. *: $p<0.05$, **: $p<0.005$.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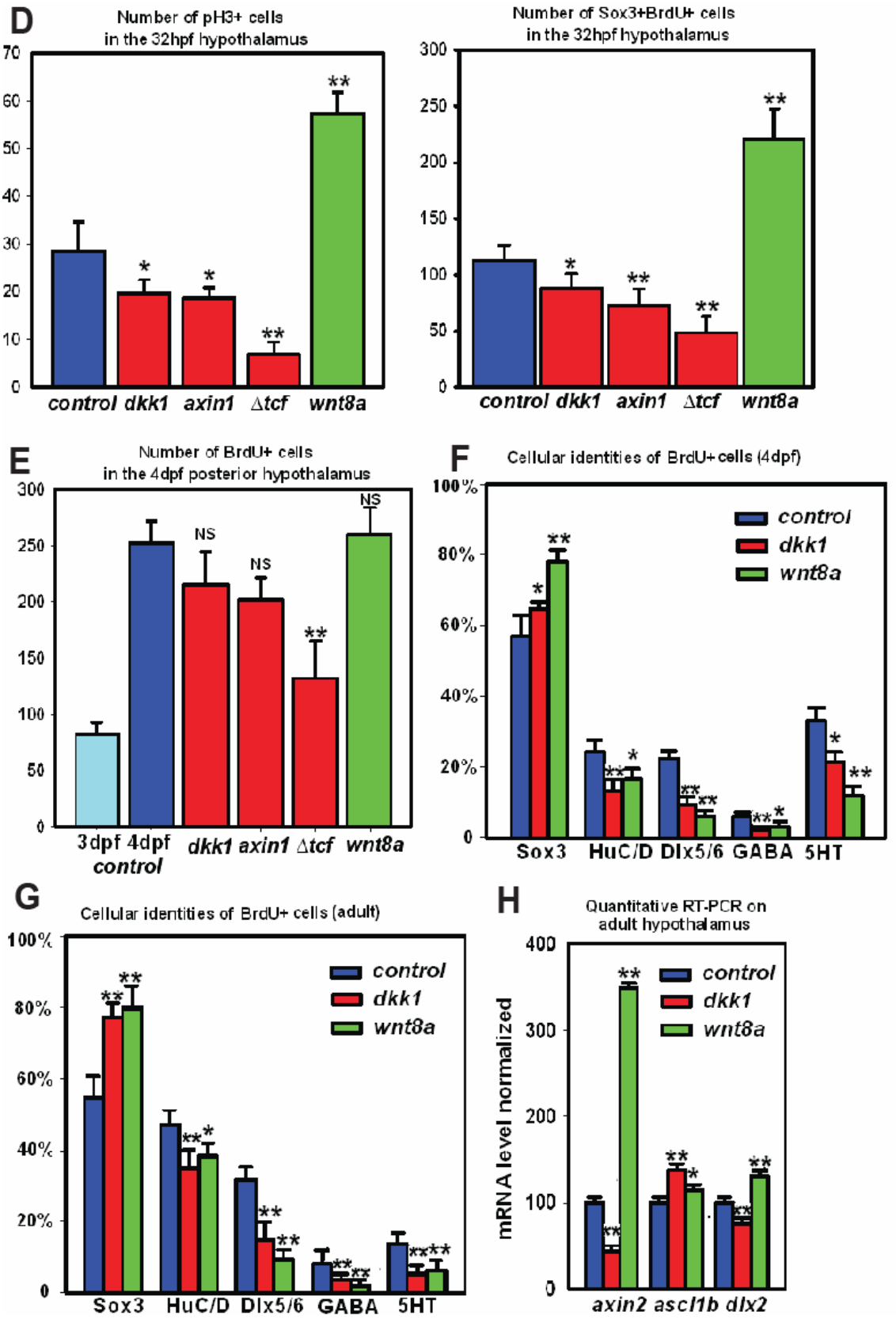


(Figure 3.3 continued)

number of $BrdU^+$ cells in the posterior recess underwent an approximate 3-fold increase over 24 hours, but we observed no statistically significant changes in proliferation following expression of *hs:wnt8a, hs:dkk1*, or *hs:axin1* (Fig. 3.3B,E). Expression of *hs:*Δ*tcf* resulted in significantly fewer $BrdU^+$ cells due to ectopic apoptosis (Supplemental Fig. 3.6). We then analyzed the $BrdU^+$ population to determine whether Wnt signaling was necessary or sufficient to promote differentiation of neural progenitors. Surprisingly, we found that both activation and inhibition of Wnt signaling caused a higher percentage of $BrdU^+$ cells to remain as undifferentiated $Sox3^+$ progenitors, and a lower percentage to differentiate into GABAergic and serotonergic progeny (Fig. 3.3B,F).

To test whether the function of Wnt signaling in neurogenesis was conserved at adult stages, we performed a similar analysis on 6-month-old zebrafish using *hs:wnt8a* and *hs:dkk1*. Because of the slower rate of proliferation at this stage, fish were incubated in BrdU for 2 days, followed by 15 daily heat-shocks. To confirm the effectiveness of transgene expression in adults, we quantified expression of the Wnt target gene *axin2* in dissected hypothalami, and found that it was significantly increased following Wnt activation and significantly decreased following Wnt inhibition (Fig. 3.3H). We then traced the $BrdU^+$ cells in the posterior recess region and found that as at 4dpf, an increased percentage remained as $Sox3^+$ neural progenitors, and fewer differentiated into mature neurons following both Wnt activation and inhibition (Fig. 3.3C,G)

Our data are consistent with a hypothesis that Wnt signaling is required for

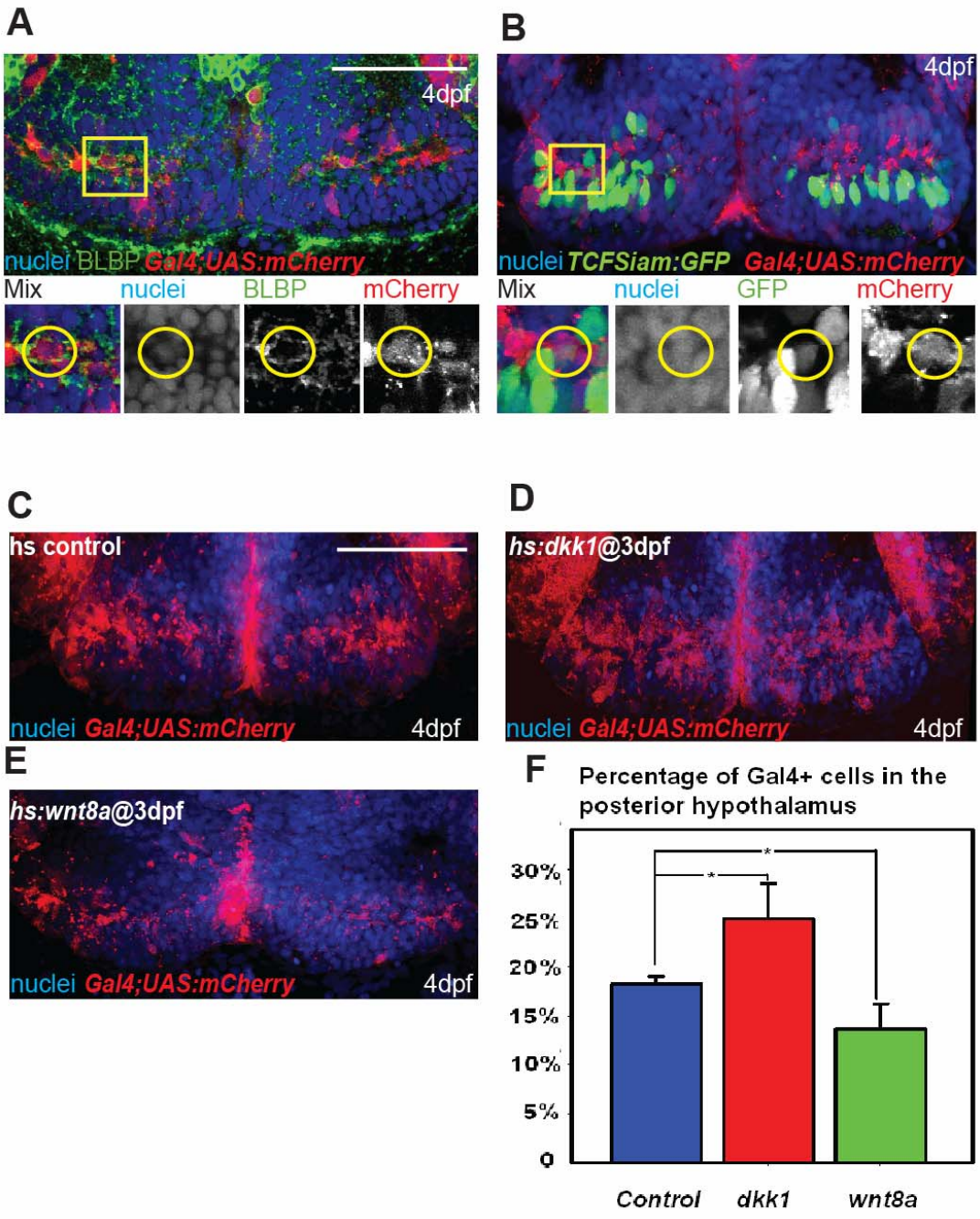
progenitors to differentiate, but must be down-regulated in order for differentiation to proceed. To test this possibility, we performed quantitative RT-PCR for neural progenitor markers in dissected adult hypothalami following heat shock. We found that the levels of *dlx2* mRNA were lower following Wnt inhibition and higher following Wnt activation, compared to control samples. In addition, we found that the levels of the proneural gene *ascl1b* were significantly higher following Wnt inhibition than in controls or following Wnt activation (Fig. 3.3H). Together, these data suggest that Wnt signaling may be required for the transition from proneural to *Dlx* gene expression, but that complete differentiation can only proceed once the pathway is no longer active.

Wnt signaling inhibits hypothalamic radial gliogenesis

Previous reports have implicated negative regulation of Wnt signaling in the maintenance of radial glia, a cell type that has been identified as a potential neural progenitor in the hypothalamus.[29, 30] We therefore asked whether the Wnt pathway was active and functional in this cell population in zebrafish. Because we did not observe any expression of GFAP in the posterior recess (Supplementary Fig. 3.7), we took advantage of a Gal4 insertion that labels radial glia expressing Brain lipid-binding protein (BLBP, Fig. 3.4A). Few $Gal4^{+}$ cells showed evidence of Wnt reporter expression at 4dpf (Fig. 3.4B), suggesting that pathway activation may not be required for their terminal differentiation or maintenance.

To test the role of Wnt signaling in radial glial development, we performed

Figure 3.4 Wnt signaling negatively regulates hypothalamic radial gliogenesis. (A) The Gal4 *zc1066a* insertion labels $BLBP^{+}$ radial-glial-like cells in the hypothalamic posterior recess at 4dpf. (B) Only a few $Gal4^{+}$ cells express the *TCFSiam:GFP* reporter at 4dpf. Boxes indicate enlarged regions, and circles indicate marker co-expression. (C-E) Gal4 *zc1066a* expression in the posterior recess of 4dpf embryos following Wnt pathway inhibition or activation at 3dpf. (F) Counts of $Gal4^{+}$ radial-glial like cells in the 4dpf hypothalamus following Wnt pathway inhibition or activation at 3dpf. Cell counts were collected from three individual samples. Scale bars: (A-E) 80μm. *: $p<0.05$.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nuclei Gal4;UAS:mCherry
4dpf
E
hs:wnt8a@3dpf
nuclei Gal4;UAS:mCherry
4dpf
F
Percentage of Gal4+ cells in the posterior hypothalamus
30%
25%
20%
15%
10%
5%
0
Control
dkk1
wnt8a

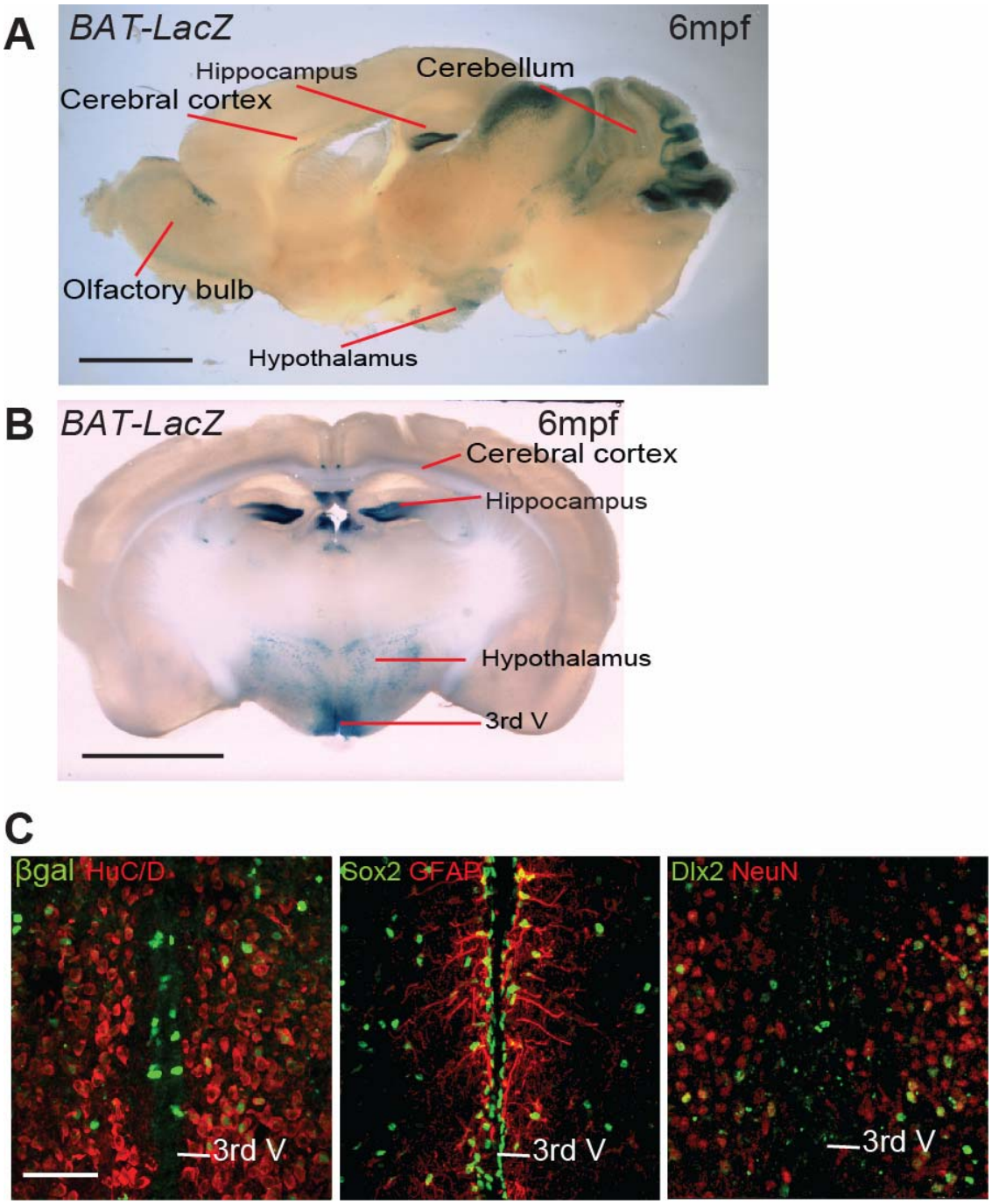
functional assays using *hs:wnt8a* and *hs:dkk1*. 72hpf embryos were heat shocked every 8 hours and fixed at 96hpf. We observed a higher density of $Gal4^+$ radial-glial like cells in *hs:dkk1* and a lower density in *hs:wnt8a* (Fig. 3.4C-F), suggesting that as in other brain regions, Wnt activity may need to be inhibited for the normal differentiation or maintenance of hypothalamic radial glia.

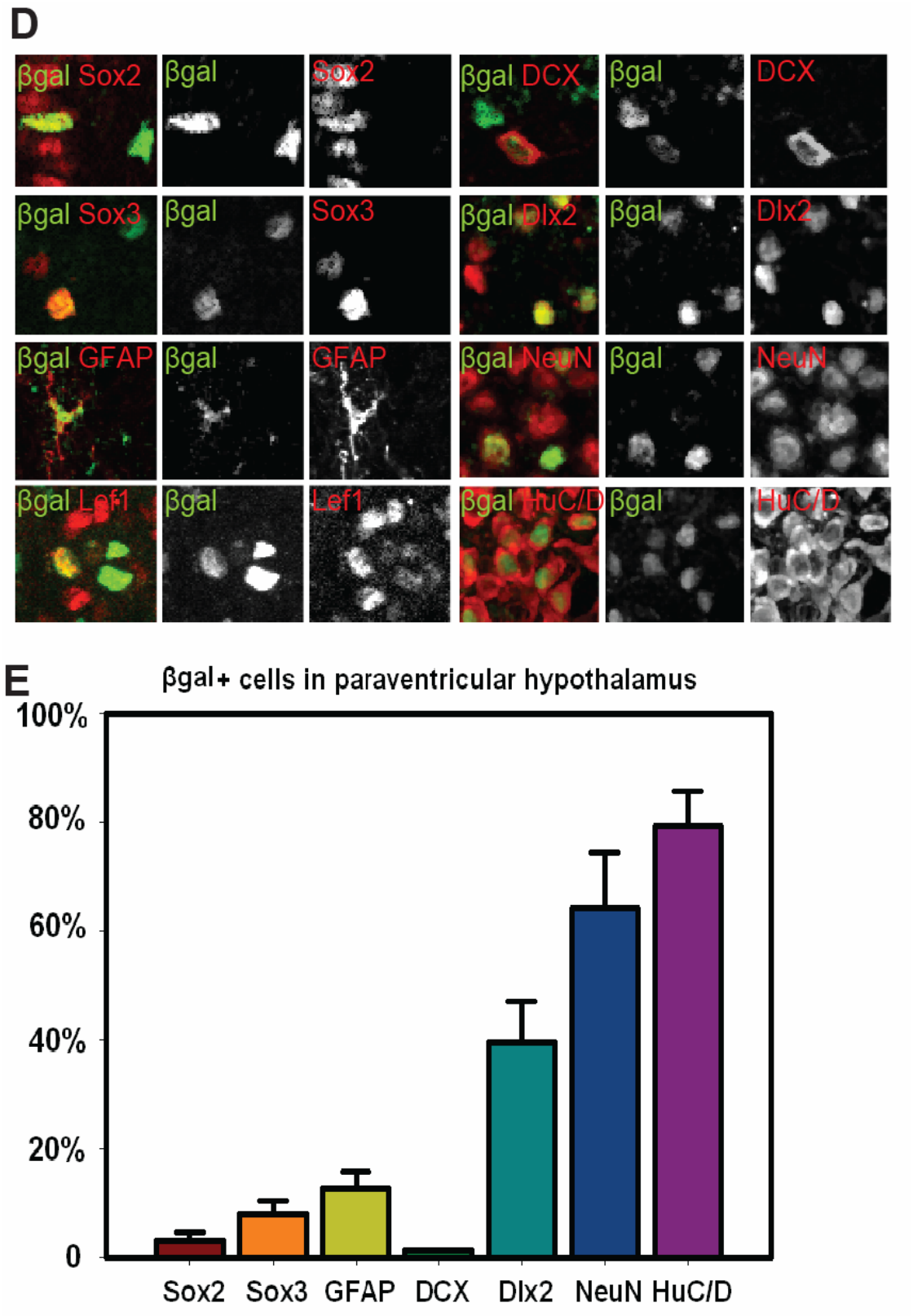
Wnt activity is present in the adult mouse hypothalamus

Zebrafish, in contrast to mammals, have widespread proliferation throughout the adult CNS.[31] Proliferating progenitors have also been identified in the rodent hypothalamus, where adult neurogenesis can influence feeding behavior.[16, 32] To determine whether the presence of Wnt signaling is conserved in the adult mouse hypothalamus, we used the reporter *BAT-LacZ* to identify Wnt-responsive cells.[33] From sagittal sections, we observed abundant ß-gal^+ cells in the adult cerebral cortex, hippocampus, olfactory epithelium, and cerebellum, as well as the posterior hypothalamus (Fig. 3.5A). From coronal sections through the hypothalamus, we determined that the paraventricular and arcuate nuclei have the highest density of Wnt-responsive cells in this region (Fig. 3.5B).

To determine the identity of hypothalamic Wnt-responsive cells we performed double immunohistochemistry for ß-galactosidase and markers of either progenitors or differentiated cell types. We found that ß-gal^+ cells exist in the ventricular and parenchymal zones of the paraventricular hypothalamus (Fig. 3.5C). In the ventricular

zone, ß-gal$^+$ cells were primarily Sox2$^+$ progenitors, and in parenchymal areas, some Figure 3.5 The adult mouse hypothalamus has a Wnt-responsive cell population. (A) Sagittal and (B) coronal sections of adult *Bat-LacZ* mouse brains. (C) ß-gal$^+$ cells are distributed in both the ventricular zone (where Sox2$^+$ and GFAP$^+$ cells reside) and the parenchymal region (where Sox2, HuC/D$^+$, NeuN$^+$, and Dlx2$^+$ cells reside). (D) Colocalization of ß-gal with specific markers in the hypothalamus. (E) Percentage of marker co-expression within the ß-gal$^+$ population. Cell counts were made from the paraventricular hypothalamus regions of six 40µm sections from 3 individual mice. Scale bars: (A-B) 2mm. (C) 80µm.


A
BAT-LacZ
6mpf
Hippocampus
Cerebellum
Cerebral cortex
Olfactory bulb
Hypothalamus
B
BAT-LacZ
6mpf
Cerebral cortex
Hippocampus
Hypothalamus
3rd V
C
βgal HuC/D
3rd V
Sox2 GFAP
3rd V
Dlx2 NeuN
3rd V



(Figure 3.5 continued)

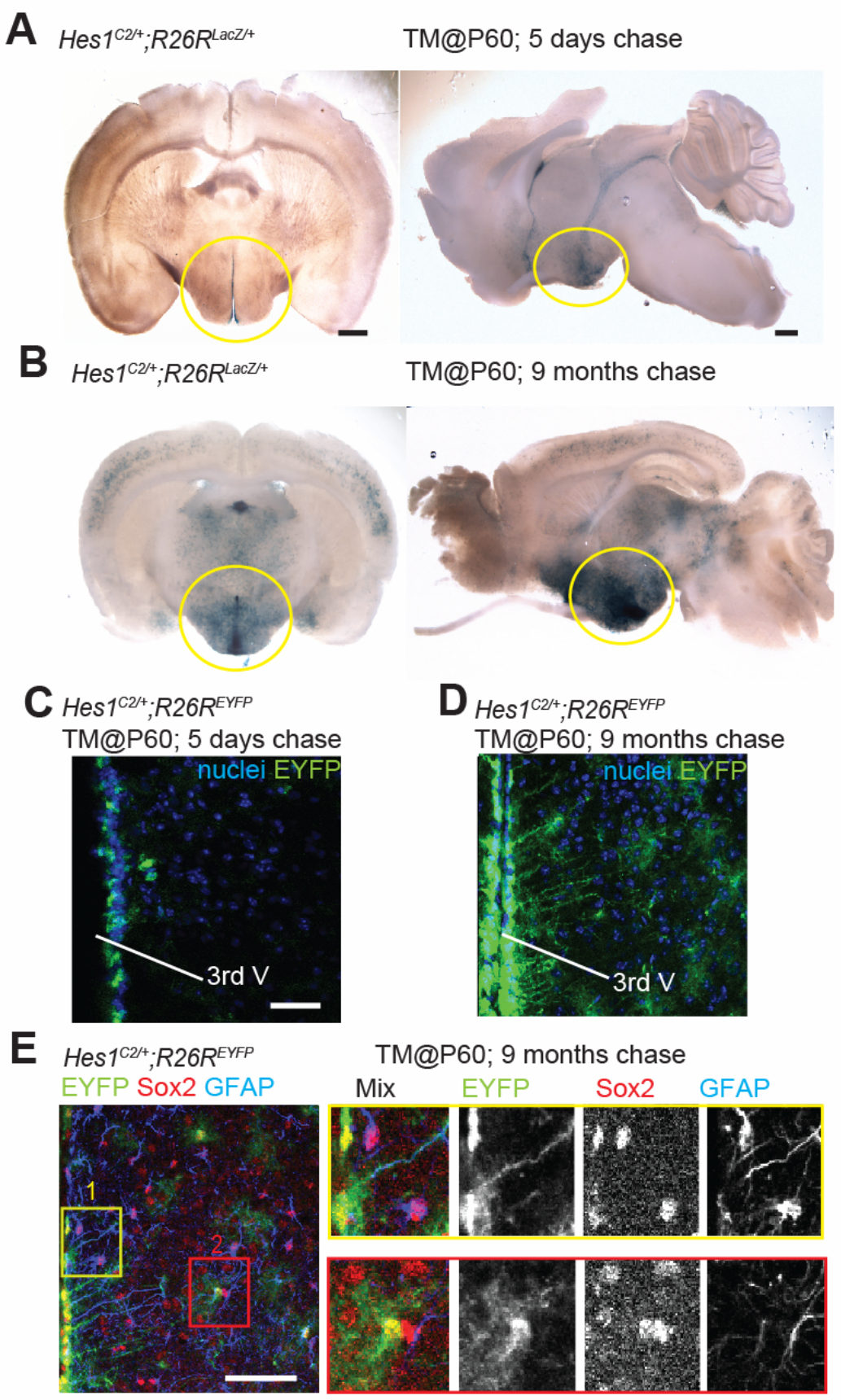
expressed markers of developing neurons and glia such as Sox3, DCX and GFAP, as well as the GABAergic precursor marker Dlx2 (Fig. 3.5D,E). The majority of parenchymal Wnt-responsive cells were HuC/D^{+} and NeuN^{+} (Fig. 3.5D,E). Together, these results suggest that Wnt-responsive cells in adult mammalian hypothalamus could be multipotent neural progenitors that contribute to adult GABAergic neurogenesis and radial gliogenesis, as in zebrafish.

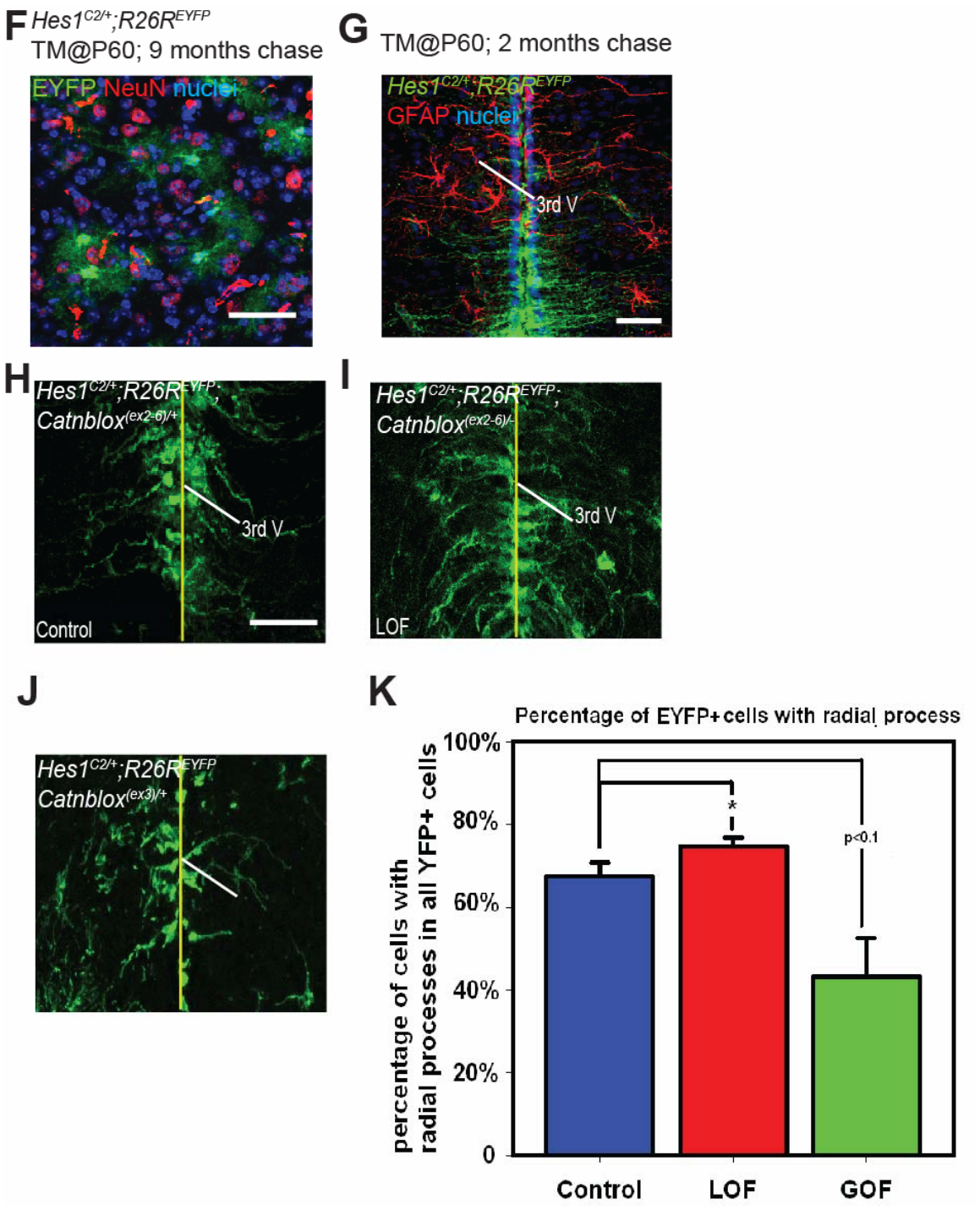
ß-catenin negatively regulates the production of ventricular hypothalamic tanycytes in the mouse

To determine the function of Wnt signaling in adult hypothalamic progenitors, we took a conditional genetic approach to remove and hyperactivate ß-catenin. We chose to trace and manipulate cells expressing the Notch effector gene *Hes1*, which functions in neural stem cell maintenance in other brain regions.[34] We observed that knock-in mice expressing tamoxifen-dependent CreERT2 recombinase from the *Hes1* locus (*Hes1*^{C2}) drive efficient and inducible recombination in hypothalamic ventricular progenitors (Fig. 3.6A,B).[35] Lineage tracing experiments with *R26R*^{LacZ} reporter mice showed that *Hes1*$^{+}$ cells are present in the ventricular zone of the adult hypothalamus 5 days after tamoxifen administration (Fig. 3.6B), followed by expansion into the parenchyma 9 months post-tamoxifen (Fig. 3.6C).[36]

To further characterize the Hes1-expressing population, we utilized the *R26R*^{EYFP} reporter in mice that received tamoxifen (TM) at P60.[37] We found that all EYFP^{+} cells

Figure 3.6 Wnt signaling inhibits the production of tanycytes from adult *Hes1*$^{+}$ progenitors. (A,B) Coronal and sagittal sections of adult *Hes1*$^{C2/+}$; *R26R*$^{LacZ/+}$ mouse brains, 5 days and 9 months after TM administration at P60. (C,D) Coronal sections through the hypothalamus of adult *Hes1*$^{C2/+}$; *R26R*$^{EYFP/+}$ mouse brains, 5 days (C) and 9 months (D) after TM administration at P60. (E,F) Co-expression of EYFP and cell type markers in *Hes1*$^{C2/+}$; *R26R*$^{EYFP/+}$ mice 9 months post-TM. Boxes in low-magnification image show enlarged ventricular (yellow) and parenchymal (red) regions. All EYFP^{+} cells are Sox2^{+}, and some ventricular EYFP^{+} cells are GFAP^{+}. No EYFP^{+} cells in parenchymal regions (F) are NeuN^{+}. (G) Co-expression of EYFP and GFAP in *Hes1*$^{C2/+}$; *R26R*$^{EYFP/+}$ mice 2 months post-TM. (H-J) Confocal projections through 40µm sections of the medial ventral paraventricular hypothalamus 2 months following ß-catenin inactivation or activation. (K) Percentage of cells with radial processes among all EYFP^{+} cells 2 months following ß-catenin inactivation or activation. Cell counts were made from hypothalamic ventricular zones on sections from three mice for each genotype. Scale bars: (A-B) 1mm. (C-J) 80µm. *: $p<0.05$.


A
Hes1C2/+;R26RLacZ/+
TM@P60; 5 days chase
B
Hes1C2/+;R26RLacZ/+
TM@P60; 9 months chase
C
Hes1C2/+;R26REYFP
TM@P60; 5 days chase
nuclei EYFP
3rd V
D
Hes1C2/+;R26REYFP
TM@P60; 9 months chase
nuclei EYFP
3rd V
E
Hes1C2/+;R26REYFP
TM@P60; 9 months chase
EYFP Sox2 GFAP
Mix
EYFP
Sox2
GFAP
1
2



(Figure 3.6 continued)

expressed Sox2 after a 5-day (data not shown), 2-month (data not shown), and 9-month chase (Fig. 3.6E), including cells located in parenchymal regions (Fig. 3.6E). The majority of EYFP$^+$ cells after a 5-day chase remained in the ventricular zone along the 3rd ventricle, having a cellular morphology without radial processes (Fig. 3.6C), while most EYFP$^+$ cells after 9 months generated radial processes that could be easily distinguished (Fig. 3.6D), only a few of which were GFAP$^+$ (Fig. 3.6E). However, we never observed any HuC/D (data not shown) or NeuN (Fig. 3.6F) expression in parenchymal EYFP$^+$ cells 9 months post TM, suggesting that *Hes1*$^+$ progenitors do not produce neurons in the adult mouse hypothalamus. Expression of these markers as well as cellular morphology indicated that the *Hes1*$^+$ lineage in the ventricular zone primarily produces glial cells including tanycytes, a class of adult radial glia that may be able to function as a neural progenitor.[18]

To investigate the role of Wnt/ß-catenin signaling in the differentiation of hypothalamic tanycytes, we crossed the *Hes1*^{C2} line with *Catnb*$^{lox(ex2\text{-}6)}$ and *Catnb*$^{lox(ex3)}$ mice allowing for ß-catenin loss- and gain-of-function experiments, respectively.[38, 39] In addition, all mice carried one copy of the *R26R*^{EYFP} reporter allele, enabling us to determine the fate of recombined *Hes1*$^+$ cells. All mice were genotyped and treated with 2 mg TM at P60, and EYFP-expressing cells in the ventral paraventricular hypothalamus were analyzed 2 months later. As at 9 months post-TM, we observed that most EYFP$^+$ cells were not GFAP$^+$ (Fig. 3.6G). We counted the number of EYFP$^+$ cells with and without radial processes, and found more EYFP$^+$ tanycytes following ß-catenin ablation

and fewer following ß-catenin activation (Fig. 3.6H-K). These results suggest that Wnt/ß-catenin activity negatively regulates the production of ventricular hypothalamic tanycytes, similar to the phenotype we observed in zebrafish.

Discussion

Wnt signaling regulates hypothalamic progenitor differentiation in zebrafish and mouse

In this study, we have shown that both the zebrafish and mouse hypothalamus contain Wnt-responsive cells throughout life. In zebrafish, hypothalamic Wnt-responsive cells are unspecified progenitors at early embryonic stages and neural progenitors at postembryonic stages. In the adult mouse, both ventricular radial glial progenitors and putative parenchymal neural progenitors exhibit Wnt activity. Hypothalamic Wnt-responsive progenitors in the two species contribute to Dlx^+ neuronal and radial glial linages, while in zebrafish they also contribute to a serotonergic lineage. Our functional analyses demonstrate that Wnt/ß-catenin signaling is required for progenitor proliferation in the early embryo, but primarily regulates progenitor differentiation at postembryonic stages. In addition, the pathway is required for progenitors to enter the proneural state, but needs to be inhibited for neurogenesis to proceed.

Transient Wnt signaling is required for GABAergic neurogenesis

Surprisingly, we found that both activation and inhibition of Wnt signaling led to arrest in the $Sox3^+$ progenitor state, and failure of further differentiation. Our data

support a model proposed previously in retinal development, in which Wnt signaling must be transiently activated in order for progenitors to acquire the competence to differentiate, but must then be inhibited for cells to complete the differentiation process.[40] Consistent with this model, we find that Wnt signaling is transiently active in postmitotic progenitors before they express *Dlx* genes. A recent study examining constitutive activation of Wnt signaling in adult neural stem cells also supports this model.[10] In our study, BrdU tracing only allowed us to follow proliferating cells, leaving open the possibility that once postmitotic progenitors have already received the necessary Wnt signal, pathway inhibition will promote their differentiation.

Contrary to other work suggesting that Wnts generally act as mitogens, we found that Wnt activity is not critically associated with proliferation at later embryonic and adult stages.[28] Within a single cell population, the function of Wnt signaling may thus change over time in a context-dependent manner. Our data suggest that while Wnt activation promotes the mitotic expansion of unspecified early progenitors, it primarily acts to hold cells in the progenitor state once they have become specified. In different cell populations, the specified state may or may not be proliferative, leading to seemingly conflicting phenotypes.

Hes1^{+} ventricular zone progenitors do not produce neurons

in the adult mouse hypothalamus

Ventricular zones have been identified as the primary adult neural stem cell niches in the vertebrate brain.[41] Using *Hes1*^{C2} lineage tracing, we observed a significant number of cells expanding outside the ventricular zone, but these cells did not become neurons. Adult hypothalamic neurogenesis in mammals has only been characterized relatively recently, partly because very few neurons are produced at any time.[42] Consistent with the idea that the mammalian hypothalamus contains a functionally significant level of adult neurogenesis during normal homeostasis, we observed numerous Dlx2^{+} cells in the subventricular and parenchymal zones. It is possible, therefore, that a novel progenitor niche separate from the ventricular zone contributes to neurogenesis, while ventricular progenitors only produce glia.[42] However we cannot rule out the alternative possibility that *Hes1*$^{-}$ neural stem cells exist in the ventricular zone and subsequently migrate to the parenchyma.[15] To test the specific role of Wnt signaling in adult mouse hypothalamic neurogenesis, it may be necessary to employ other genetic tools, such as inducible Cre-mediated drivers localized specifically to Wnt-responsive cells or to progenitors in the GABAergic lineage.

The role of Wnt signaling in hypothalamic radial glia

While tanycytes have been shown to proliferate following growth factor administration *in vivo* and can produce neurospheres *in vitro*, their developmental

capabilities during normal homeostasis are unknown.[14, 30] Our data suggest tanycyte formation and maintenance does not require Wnt signaling. In contrast, we find that Wnt activation leads to decreased tanycyte numbers and changes in morphology. Other work has revealed a similar role for ß-catenin in Bergmann glia, the resident population in the cerebellum.[29] While our experiments were not able to distinguish between defects in tanycyte development and loss of these cells from transdifferentiation, they nevertheless indicate that low Wnt activity plays a fundamental role in the continued presence of this important cell type.

In summary, this work may help resolve some of the seemingly conflicting conclusions regarding the role of Wnt signaling in neurogenesis, from studies in different systems using different manipulations. We believe that many of these results can be explained by a dual role for the pathway, first in expansion of unspecified progenitors, then later as a transiently required inducer of neuronal differentiation. Furthermore, we have established the vertebrate hypothalamus as a new model system for Wnt function in postembryonic progenitor differentiation, and opened the field to future studies examining the role of this process in animal behavior.

Experimental Procedures

Fish strains and embryo manipulations

Embryos were obtained from natural spawning of wild-type (AB*), transgenic, and mutant adult fish. The following lines have been described previously: *Tg(TOP:GFP)*^{w25},

Tg(gfap:GFP)^{mi2001}, *Tg(hsp701:tcf3-GFP)*^{w26}, *Tg(hsp701:wnt8a-GFP)*^{w34}, *Tg(hsp701:dkk1-GFP)*^{w32}, *Et(T2KHG)*^{nkhg21c}, *Tg(1.4dlx5a/-dlx6a:GFP)*^{ot1}, and *Tg(UAS-E1b:NfsB-mCherry)*^{jh17}.[45, 19, 20, 22, 26, 27, 43, 44]

lef1^{zd11} was made by ZFN mutagenesis (described in detail below).

Tg(hsp701:GFP-axin1)^{zd13} was made by inserting a cDNA containing *GFP* fused to zebrafish *axin1* into a pCS2+ backbone vector containing the *hsp70-4* promoter. A stable transgenic line was produced by plasmid injection at the 1-cell stage followed by screening for germline transmission of heat shock-induced GFP expression.

Tg(dlx5/6:mCherry)^{zd14} was made by inserting the *1.4dlx5a/-dlx6a* enhancer/promoter [44] upstream of an *mCherry* cDNA in a Tol2 destination vector using multisite Gateway cloning.[44, 46] A stable transgenic line was produced by plasmid injection with *tol2* mRNA at the 1-cell stage followed by screening for germline transmission of mCherry expression.

Tg(7xTCF-Xla.Siam:GFP)^{ia4} (short name *TCFSiam:GFP*) was made by inserting the 7xTCF-Siam enhancer/promoter upstream of an *eGFP* cDNA in a Tol2 destination vector using multisite Gateway cloning.[33, 46] A stable transgenic line was produced by plasmid injection with *tol2* mRNA at the one-cell stage followed by screening for germline transmission of GFP expression.

The *Et(Gal4VP16; myl7:gfp)*^{zc1066a} enhancer-trap line was generated by plasmid injection with *tol2* mRNA at the one-cell stage. Potential founders were crossed to *Tg(UAS-E1b:Kaede)*^{s1999t} fish for testing and identified by Kaede expression in embryos.

Identified F1 transgenics were crossed to *Tg(UAS-E1b:nfsB-mCherry)*^{jh17} fish and embryos were imaged at 1, 2, and 5 dpf for identification of expression patterns.

Adult brains were dissected from anesthetized fish fixed in 4% paraformadehyde for 2 days. Fish received BrdU pulses by immersion in E3 media with 10mM BrdU, and received heat shock by immersion in prewarmed E3 media.

Mice

BAT-LacZ, *Hes1*^{C2}, *R26R*^{EYFP}, *R26R*^{LacZ}, and *Ctnnb1*$^{lox(ex3)}$ mice have been described previously. [33, 35-37, 39] Floxed and germline β-catenin loss-of-function mice, Ctnnb1^{lox} and Ctnnb1$^{\Delta}$ respectively, were obtained from Jackson Laboratories.[38] *Hes1*$^{C2/+}$*; R26R*$^{LacZ/+}$ mice were used for lineage tracing experiments. For ß-catenin loss-of-function experiments, *Hes1*$^{C2/+}$*; R26R*$^{EYFP/+}$*; Ctnnb1*$^{lox/\Delta}$ mice (referred to as *Ctnnb1*$^{Hes1\text{-}cKO}$) were compared to control *Hes1*$^{C2/+}$*; R26R*$^{EYFP/+}$*; Ctnnb1*$^{lox/+}$ (*Ctnnb1*$^{Hes1\text{-}het}$) animals. To assess a potential ß-catenin gain-of-function phenotype, crosses were set up to yield *Hes1*$^{C2/+}$*; R26R*$^{EYFP/+}$*; Ctnnb1*$^{lox(ex3)/+}$ (*Ctnnb1*$^{Hes1\text{-}GOF}$) and *Hes1*$^{C2/+}$*; R26R*$^{EYFP/+}$ (*Ctnnb1*$^{Hes1\text{-}ctrl}$) mice. All genotypes included the *R26R*^{EYFP} or *R26R*^{LacZ} reporter allele to follow the fate of recombined cells. Tamoxifen (Sigma T-5648) was dissolved in corn oil, and administered by oral gavage at doses of 10 mg (Hes1 lineage) or 2 mg (ß-catenin loss and gain of function experiments) per mouse between 6-8 weeks of age. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Utah.

X-gal staining, in situ hybridization and immunohistochemistry

For X-gal staining in mice, fixed whole brains were sliced at 500μm thickness, and staining was performed as described previously followed by clearing in 100% glycerol.[47] Mouse immunostaining and analysis were performed as described previously [35]. Zebrafish in situ hybridization and immunohistochemistry were performed as described previously, except that whole mount brains were first dissected (4dpf and adult) and sliced (adult), followed by collagenase treatment.[48] All mRNA probes have been described previously.[11, 12]

Primary antibodies used were: mouse anti PCNA (Sigma: P8825), rabbit anti Sox3 (Gift from M. Klymkowsky), rabbit anti GFP (Molecular Probes: A11122), mouse anti GFP (Molecular Probes: A11120), chick anti GFP (Aves Labs: GFP-1020), mouse anti HuC/D (Molecular Probes: A21271), rabbit anti 5HT (ImmunoStar: 541016), rabbit anti GABA (Sigma: A2052), mouse anti BrdU (Sigma: B8434), rat anti BrdU (Abcam: ab6326), rabbit anti BLBP (Abcam: ab32432), mouse anti GFAP (zrf-1; ZIRC [Eugene, OR]), goat anti Sox2 (Santa Cruz: sc-17320), rabbit anti DCX (Abcam: ab18732), rabbit anti Dlx2 (Abcam: ab18188), rabbit anti GFAP (Abcam: ab7260), mouse anti NeuN (Millipore: MAB377), chick anti LacZ (Abcam: ab9361), rabbit anti pH3 (Cell Signaling: 9713), rabbit anti Lef1 (Open Biosystems), rabbit anti LEF1 (Cell Signaling: 2230). Secondary antibodies were obtained from Jackson ImmunoResearch. Hoechst33342 was used to stain cell nuclei.

Cryosectioning and microscopy

Cryosections were cut at a thickness of 12µm for embryonic zebrafish and 30-40µm for adult mouse brains. Fluorescent images of whole mount brains were taken using an Olympus FV1000 confocal microscope and a fluorescent dissecting microscope. Bright field images were obtained using a conventional compound microscope.

Data analysis

Colocalization analysis, volume measurements, statistical calculations, and graphs were generated with Image J, Volocity5.4, Amira 5.3.3, Microsoft Excel, and Sigma Plot 10.0. Error bars represent standard deviations from at least three samples, and comparisons of data sets were performed with one-tailed homoscedastic or heteroscedastic t-tests.

Quantitative RT-PCR

Total RNA was isolated using an RNeasy extraction kit (Qiagen) followed by DNase treatment. cDNA was synthesized by SuperScript II reverse transcriptase (Invitrogen). All mRNA levels were normalized to an average of *beta-actin* and *ribosomal protein L*, in order to account for changes in cell number. Quantitative realtime PCR using the Sybr Green reagent was performed with an ABI7900, using primers listed below:

axin2:

F: TGAAGCGGGAACAGGAAAC; R: AGGAGCAAAGGCAGAGAA.

ascl1b:

F: GGAGTGTAAAGTGCTGAAGAGA; R: TGCTGCTGAGGGATTGTGT.

dlx2:

F: GCAACAGAACTCAGAAAGCCTAC; R: AAAGCAAGTCCAACGACAGAG.

rpl12:

F: GTCTCCCTTCTTGGGCTTAG; R: CATTGGCATCTCTGTTGACTC.

beta-actin:

F: AGGATGCGGAAACTGGAAAG; R: GAGGAGGGCAAAGTGGTAAA.

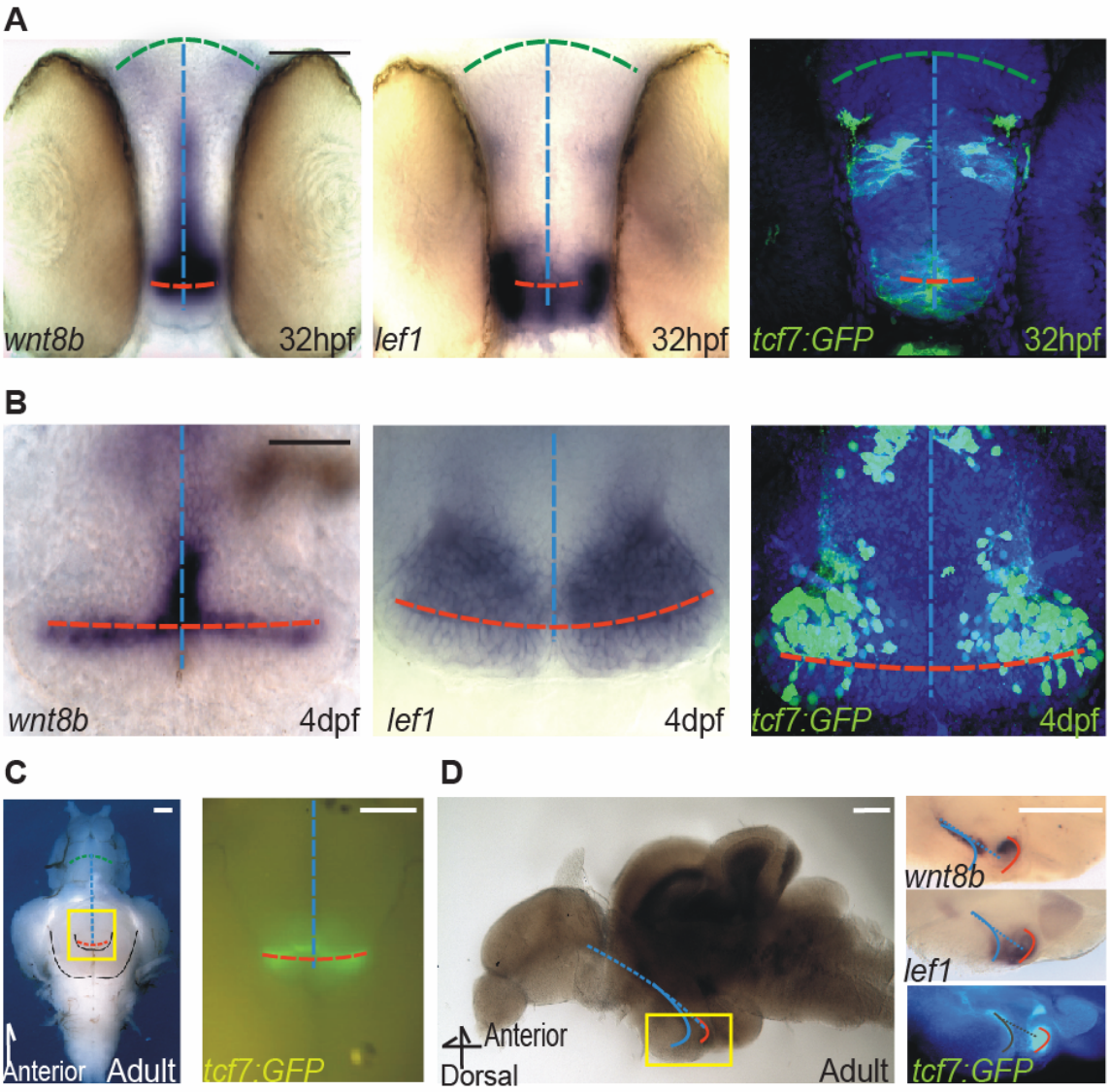
Isolation of *lef1* mutants using engineered zinc-finger nuclease

To introduce mutations in the *lef1* gene, a target site was chosen within the exon 1 using ZiFiT (http://zifit.partners.org/ZiFiT/). ZFNs were prepared using a detailed protocol posted elsewhere (http://wiki.zfin.org/), in which three-finger array libraries were constructed using OPEN pools, but modified to be selectable in bacterial one-hybrid (B1H) system.[49, 50] Selected three-finger array sequences were converted to ZFNs by fusion with the FokI nuclease domain. Synthetic mRNAs encoding the ZFNs were injected into one-cell stage zebrafish embryos. Mutations were identified by loss of BsaJI restriction sequence located in the target site. Genomic DNA was extracted from the individual 24 hpf embryos and amplified with the following primers:

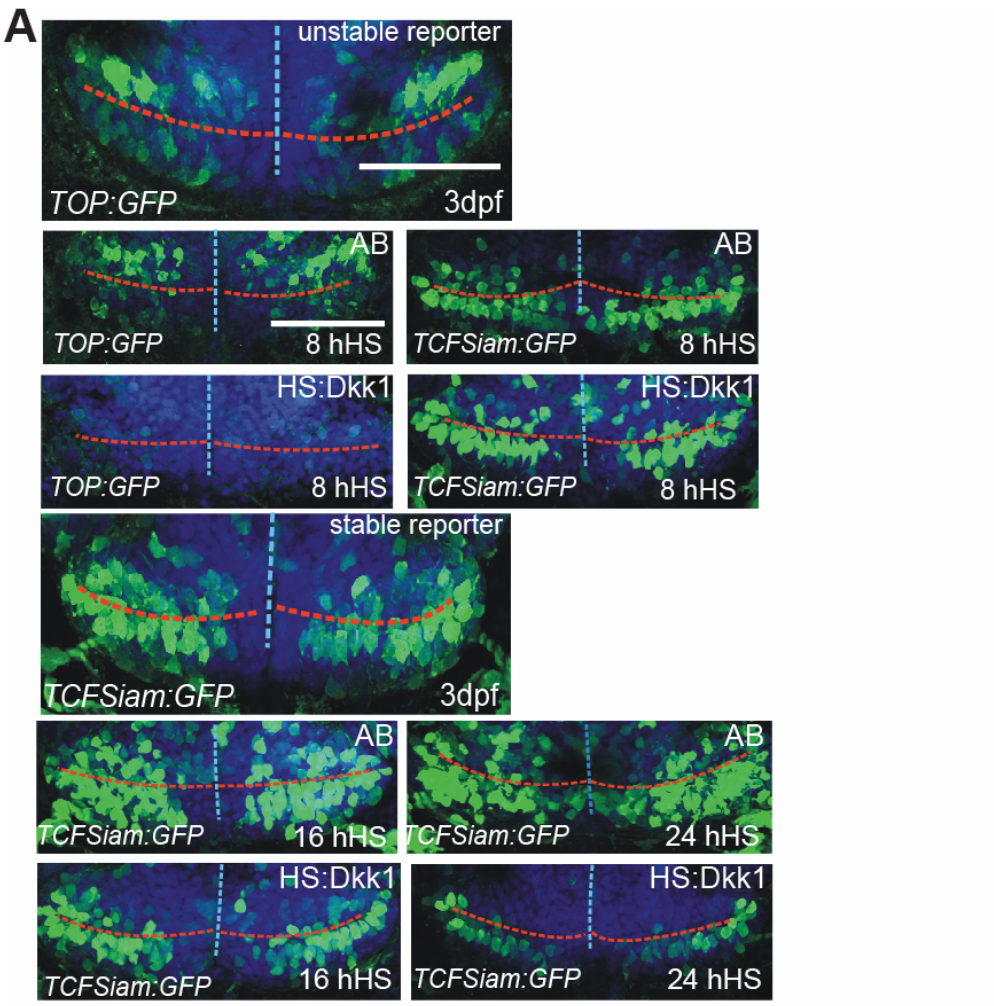
F: TTGGAGGTGTGCTACTCACG; R: CACTCTCTCCAGCCCAACAT.

To isolate germ-line transmitted *lef1* mutations, ZFN-injected embryos were raised to adulthood and progeny were analyzed using PCR and BsaJI digestion.

Supplementary Figure 3.1 Expression of *wnt8b*, *lef1*, and *tcf7:GFP* at 32hpf, (A) and 4dpf (B) in the zebrafish hypothalamus from ventral whole-mount views. (C) Bright-field and fluorescent views of the adult hypothalamus expressing *tcf7:GFP*. Yellow box in the bright field image indicates the region magnified in the fluorescent image. (D) Sagittal view of *wnt8b*, *lef1* and *tcf7:GFP* expression in the adult hypothalamus. Yellow box in the left image indicates the region magnified in the right images. Blue line: 3rd ventricle; Red line: Posterior recess. Scale bars: (A-B) 50µm, (C-D) 250µm.


A
wnt8b
32hpf
lef1
32hpf
tcf7:GFP
32hpf
B
wnt8b
4dpf
lef1
4dpf
tcf7:GFP
4dpf
C
Anterior
Adult
tcf7:GFP
D
Anterior
Dorsal
Adult
wnt8b
lef1
tcf7:GFP

Supplementary Figure 3.2 Two Wnt reporter lines respond differently to Wnt pathway inhibition. (A) Ventral view of the posterior recess region of *TOP:GFP* and *TCFSiam:GFP* embryos, 8hours, 16hours, and 24hours after *hs:dkk1* activation at 3dpf. Images are 50μm confocal projections. (B) Relative percentages of GFP^{+} cells compared to wild-type controls. Counts were made from at least three individual samples for each condition. Error=±SD. Scale bar: 80μm.


A
unstable reporter
TOP:GFP
3dpf
AB
TOP:GFP
8 hHS
AB
TCFSiam:GFP
8 hHS
HS:Dkk1
TOP:GFP
8 hHS
HS:Dkk1
TCFSiam:GFP
8 hHS
stable reporter
TCFSiam:GFP
3dpf
AB
TCFSiam:GFP
16 hHS
AB
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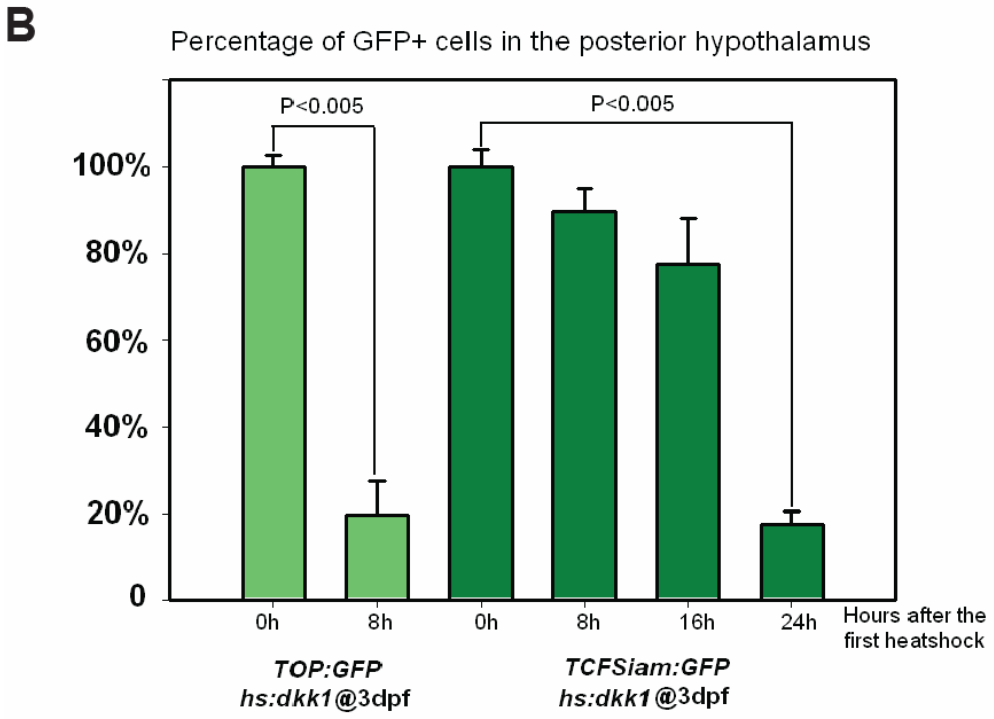
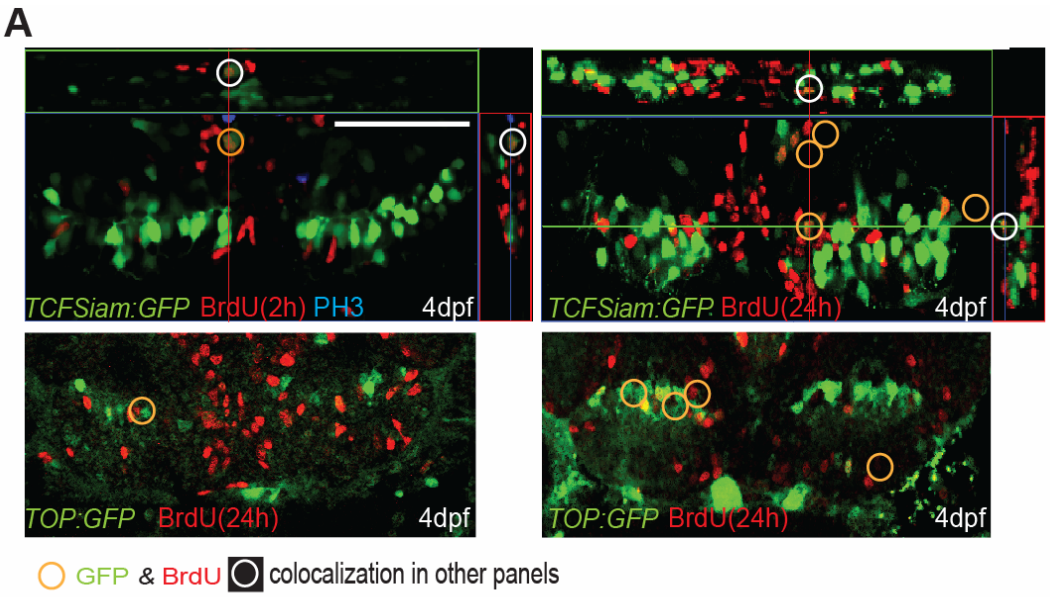
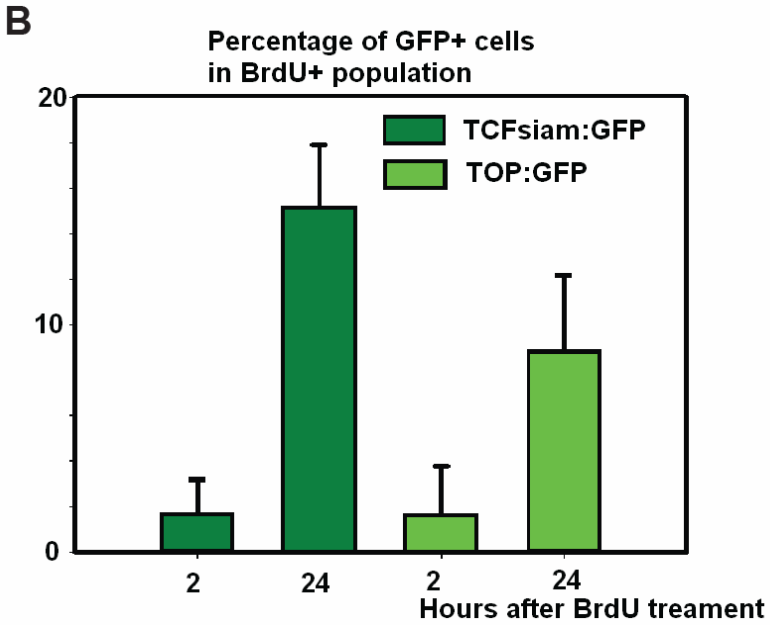
16 hHS
HS:Dkk1
TCFSiam:GFP
24 hHS

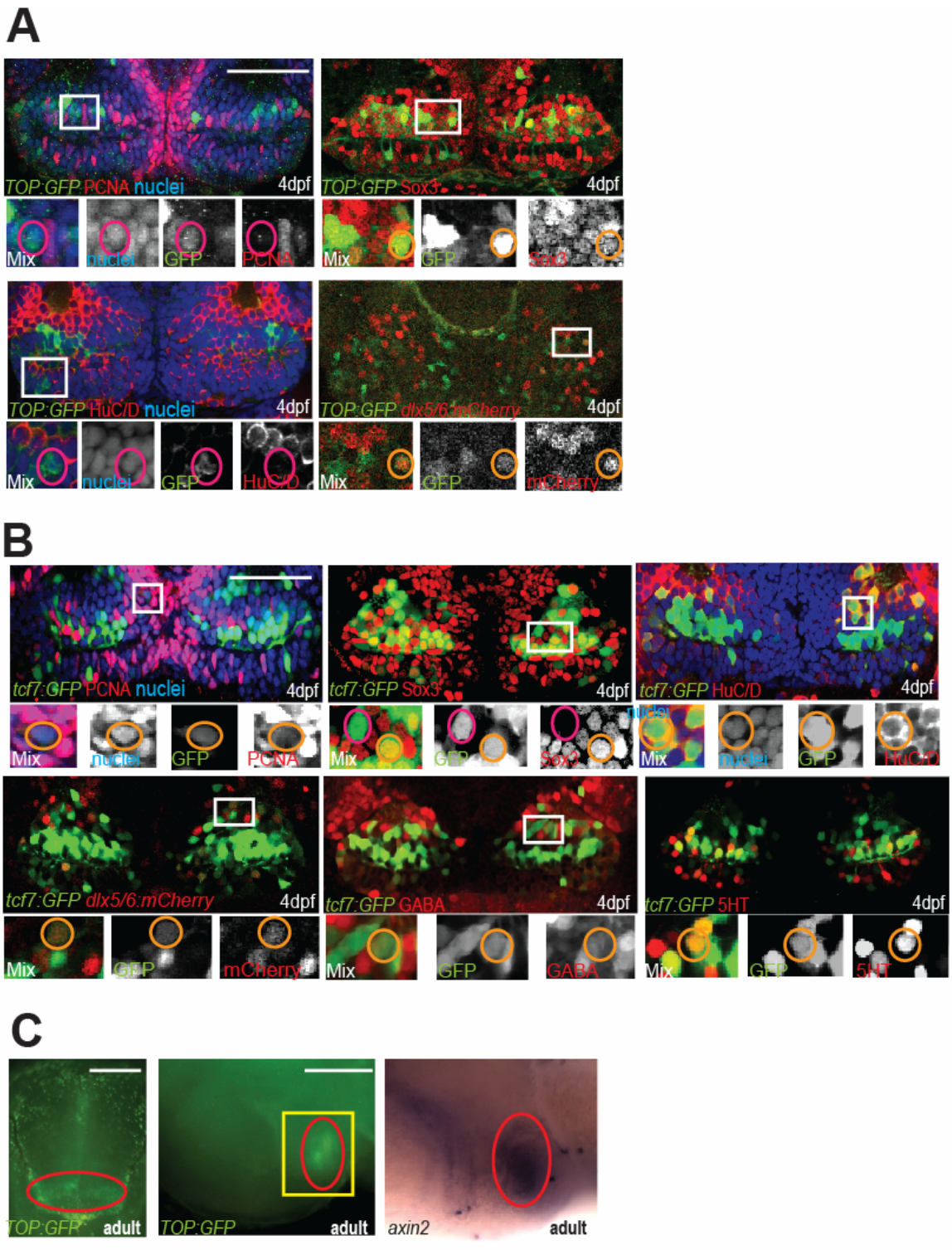

B
Percentage of GFP+ cells in the posterior hypothalamus
P<0.005
P<0.005
100%
80%
60%
40%
20%
0
0h
8h
0h
8h
16h
24h
Hours after the first heatshock
TOP:GFP
hs:dkk1@3dpf
TCFSiam:GFP
hs:dkk1@3dpf

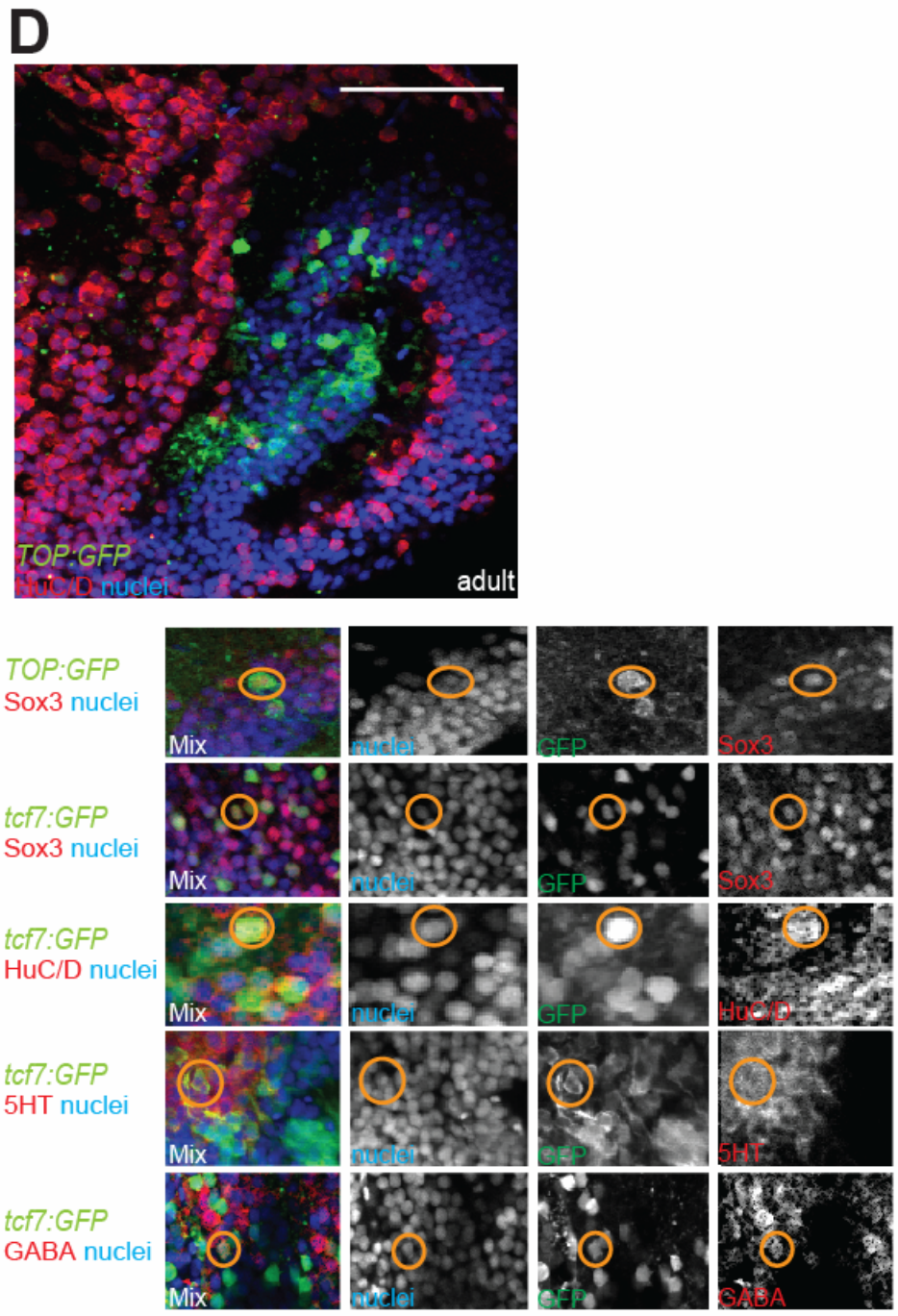
Supplementary Figure 3.3 Few Wnt-responsive cells are proliferating at 4dpf. (A) Ventral view of the posterior recess region of *TOP:GFP* and *TCFSiam:GFP* hypothalamus after short (2h) and long term (24h) BrdU labeling; observed with 50µm confocal projections. (B) Quantification of $BrdU^{+}$ reporter-expressing cells. Counts were made from at least 3 individual samples for each condition. Error=±SD. Scale bar: 80µm.


A
TCFSiam:GFP BrdU(2h) PH3 4dpf
TCFSiam:GFP BrdU(24h) 4dpf
TOP:GFP BrdU(24h) 4dpf
TOP:GFP BrdU(24h) 4dpf
GFP & BrdU colocalization in other panels


B
Percentage of GFP+ cells
in BrdU+ population
20
10
0
TCFsiam:GFP
TOP:GFP
2
24
2
24
Hours after BrdU treament

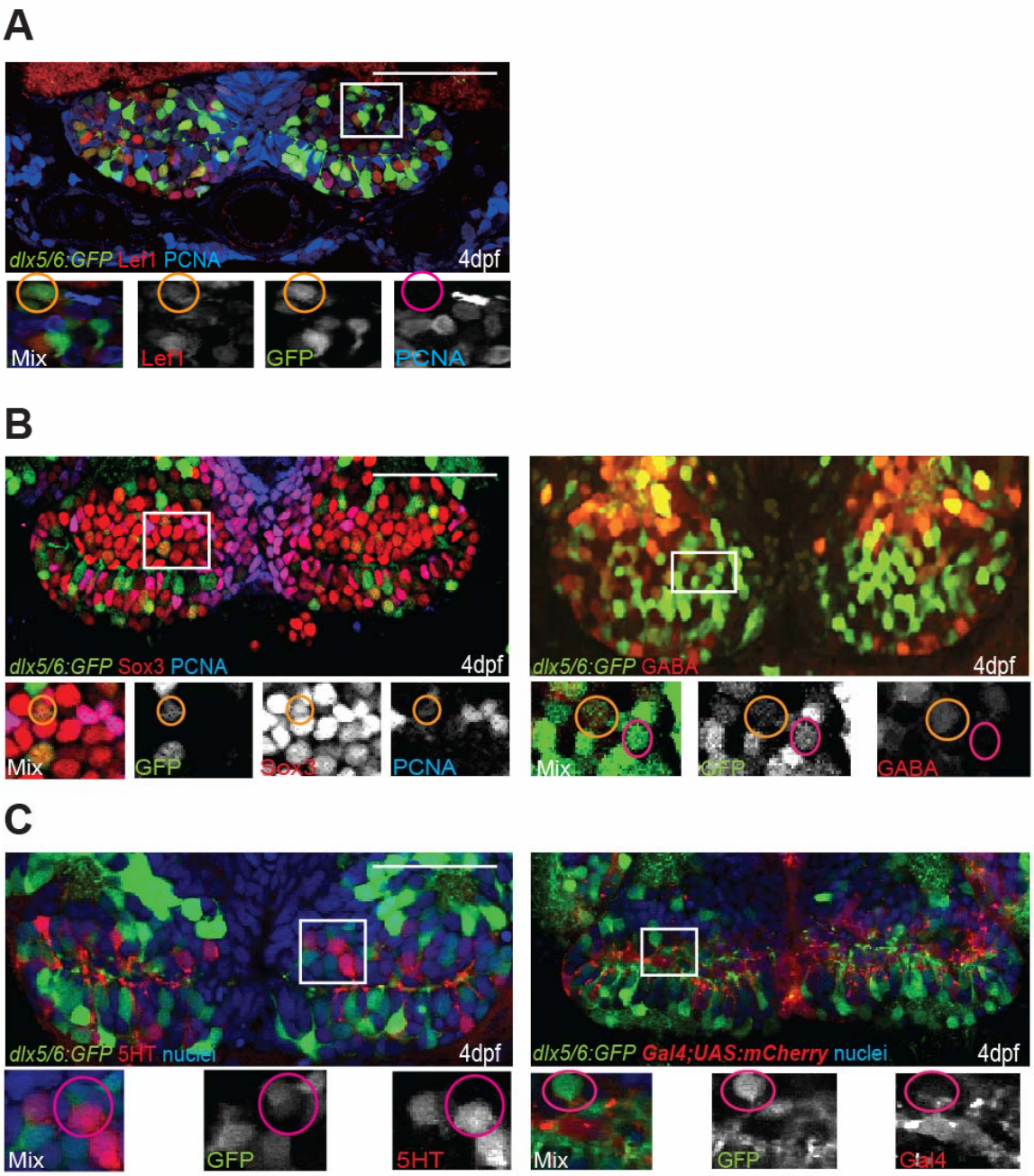
Supplementary Figure 3.4 Identity of *TOP:GFP* and *tcf7:GFP*$^{+}$ cells. (A-B) Co-staining of GFP in the 4dpf hypothalamus with cell-type specific markers. (C-D) Co-staining of GFP in the adult hypothalamus with cell-type specific markers. White boxes indicate enlarged regions. Small orange circles label cells with colocalization and small magenta circles label cells without colocalization. Scale bars: (A, B, D) 80μm, (C) 250μm.

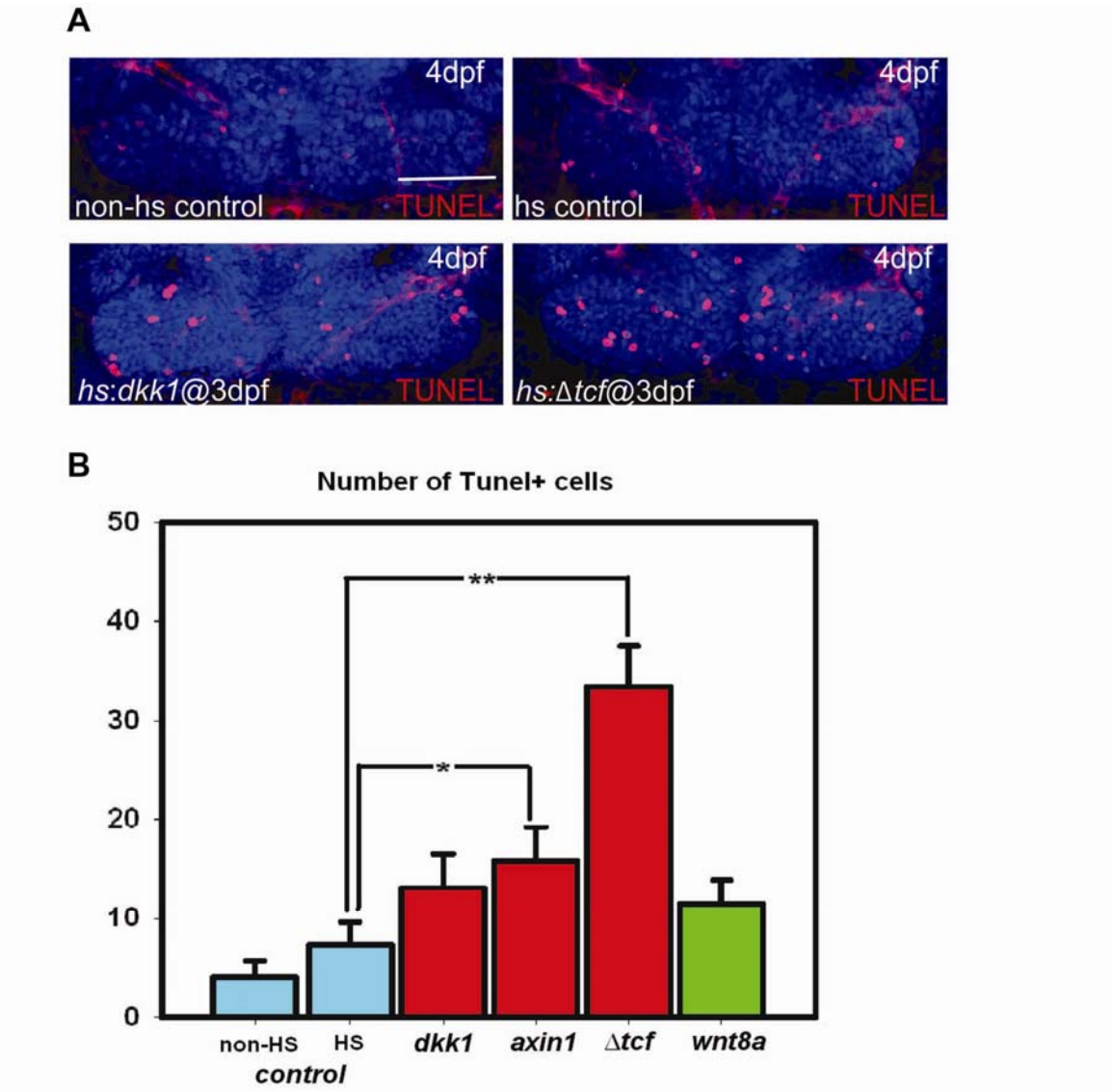

A
TOP:GFP PCNA nuclei
4dpf
TOP:GFP Sox3
4dpf
Mix
nuclei
GFP
PCNA
Mix
GFP
Sox3
TOP:GFP HuC/D nuclei
4dpf
TOP:GFP dlx5/6:mCherry
4dpf
Mix
nuclei
GFP
HuC/D
Mix
GFP
mCherry
B
tcf7:GFP PCNA nuclei
4dpf
tcf7:GFP Sox3
4dpf
tcf7:GFP HuC/D
4dpf
Mix
nuclei
GFP
PCNA
Mix
GFP
Sox3
Mix
nuclei
GFP
HuC/D
tcf7:GFP dlx5/6:mCherry
4dpf
tcf7:GFP GABA
4dpf
tcf7:GFP 5HT
4dpf
Mix
GFP
mCherry
Mix
GFP
GABA
Mix
GFP
5HT
C
TOP:GFP
adult
TOP:GFP
adult
axin2
adult



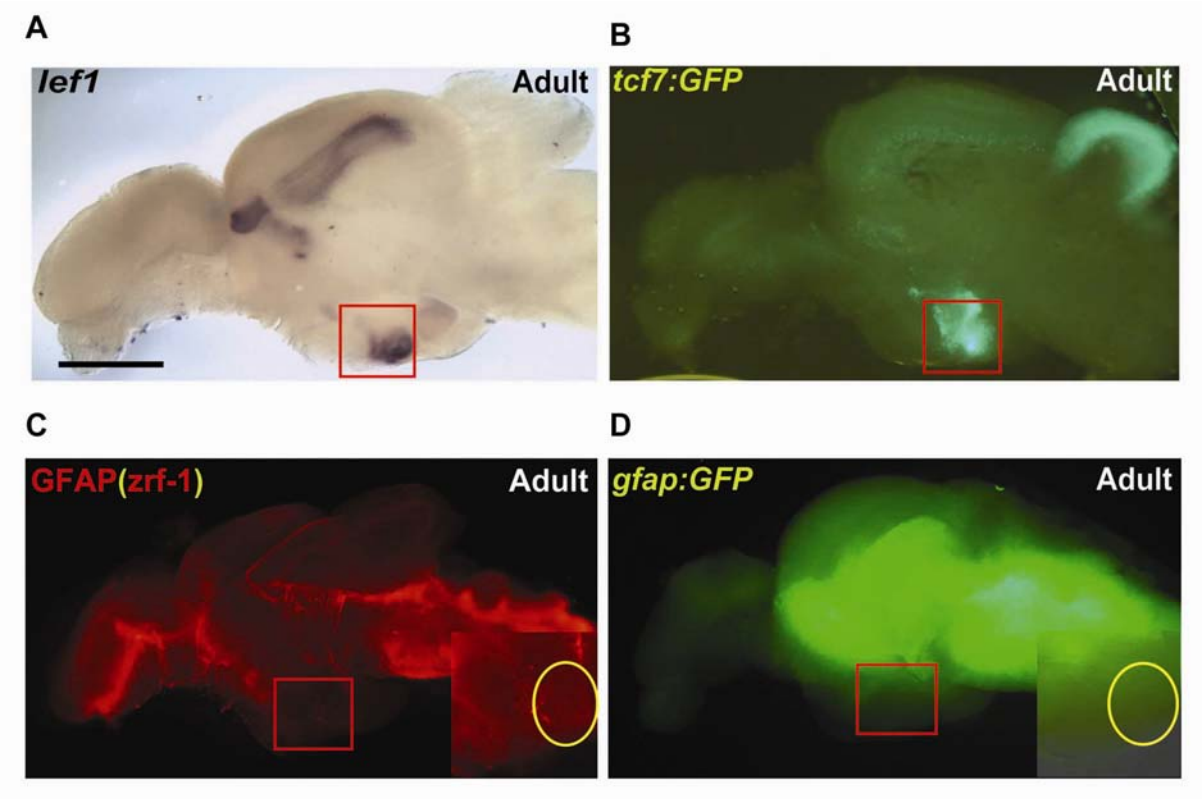
(Supplementary Figure 3.4 continued)

Supplementary Figure 3.5 Identity of *dlx5/6:GFP* cells. (A,B) Co-labeling of *dlx5/6:GFP* with cell-type specific markers. *dlx5/6:GFP* expression overlaps with Sox3, PCNA, Lef1, and GABA, but not with 5HT or a Gal4 insertion expressed in radial glia (C). White boxes indicate enlarged regions. Small orange circles label cells with colocalization and small magenta circles label cells without colocalization. Scale bars: 80μm.


A
dlx5/6:GFP Lef1 PCNA
4dpf
Mix
Lef1
GFP
PCNA
B
dlx5/6:GFP Sox3 PCNA
4dpf
Mix
GFP
Sox3
PCNA
dlx5/6:GFP GABA
4dpf
Mix
GFP
GABA
C
dlx5/6:GFP 5HT nuclei
4dpf
Mix
GFP
5HT
dlx5/6:GFP Gal4;UAS:mCherry nuclei
4dpf
Mix
GFP
Gal4



Supplementary Figure 3.6 Cell death following manipulation of Wnt signaling at 3-4dpf. (A) TUNEL staining; (B) Counts of TUNEL^{+} cells. Only *hs:*Δ*tcf* and *hs:axin1* (to a lesser extent) significantly increase cell death. Scale bar: 50μm



Supplementary Figure 3.7 The posterior recess of the adult zebrafish hypothalamus does not express GFAP. Whole mount mid-sagittal views of adult brains are shown: (A-B) *lef1* and *tcf7:GFP* are expressed strongly in the posterior hypothalamus. (C-D) The radial glial and astrocyte marker GFAP as well as a *gfap:gfp* transgene are not expressed in the posterior hypothalamus. Scale bar: 800µm.

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CHAPTER 4

DISCUSSION

Progress made by my work

My studies stemmed from the observation that the post-embryonic vertebrate hypothalamus may contain active Wnt-responsive cells and adult neural progenitors.[1] When I initially began my studies, it was unknown which Wnt components contributed to adult hypothalamic Wnt activity, and the identity of Wnt-responsive cells as well as the precise role of Wnt signaling in hypothalamic neurogenesis were unclear. My thesis work answered these questions.

With the help of fellow graduate student Robert Duncan, I performed expression analyses of all TCF family members and Wnt ligands, and identified *lef1*, *tcf7*, and *wnt8b* as the strongest candidates responsible for embryonic and post-embryonic Wnt activity in the zebrafish hypothalamus. I also found expression of *axin2* and *wnt inhibitory factor* (*WIF*) in the posterior hypothalamus (data not shown). Together, these data provide the most complete genetic atlas illustrating hypothalamic Wnt signaling (Fig. 4.1).

I also performed substantial immunohistochemical analysis of two Wnt reporter lines, *TOP:dGFP* and *7*TCFsiam:GFP*, comprehensive analysis of a *tcf7:GFP* transgenic line, and Lef1 expression analysis via antibody staining. I found that the identities of hypothalamic Wnt-responsive cells changed over time: at early embryonic stages (32hpf), they are $PCNA^{+}$ progenitors expressing no lineage markers, suggesting they are pluripotent stem cells; however, by 4dpf and into adulthood, they are Sox3+/$PCNA^{-}$ neural progenitors. Through lineage analysis I also elucidated what cell types these Wnt-responsive progenitors eventually generate: GABAergic, dopaminergic, and

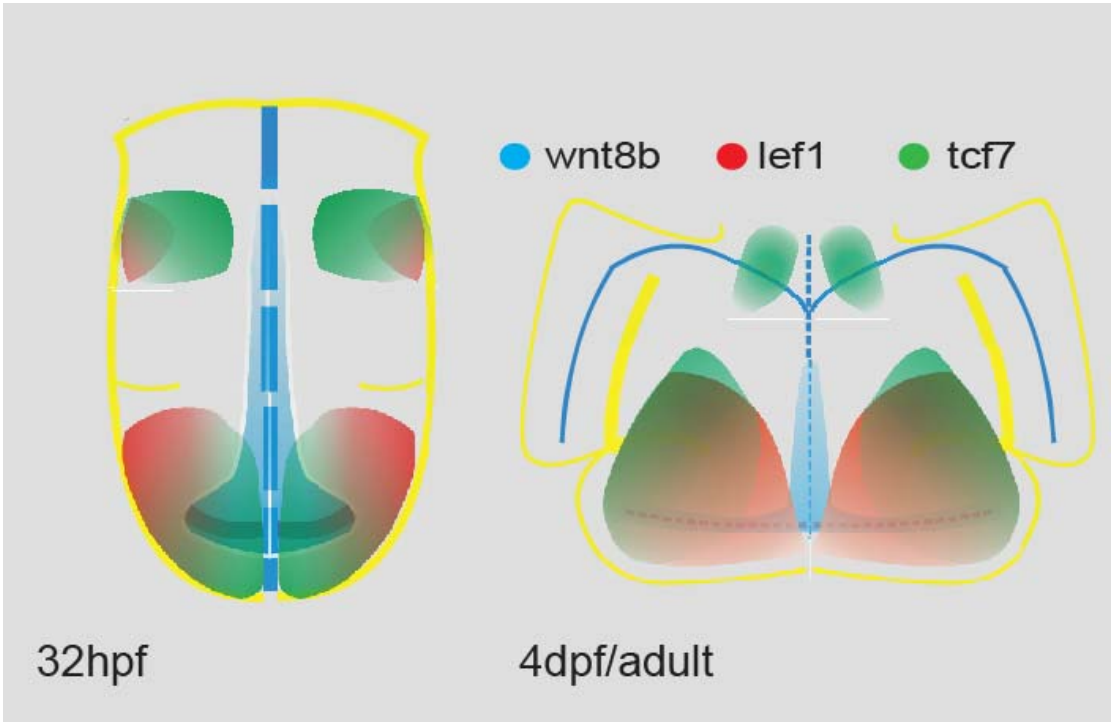

Figure 4.1 The expression pattern of *wnt8b*, *lef1* and *tcf7* in ventral views of the zebrafish hypothalamus

serotonergic neuronal populations, as well as a subset of the tanycyte population. My data suggest that the contributions of Wnt-responsive cells to GABAergic neurons and tanycytes are evolutionarily conserved between zebrafish and mouse.

I propose that Wnt plays a dynamic role in hypothalamic neurogenesis: during early embryogenesis Wnt signaling acts as a general mitogen that maintains the normal size of the hypothalamic progenitor pool. However, at 4dpf and into adulthood, Wnt signaling is not critically required for proliferation. Instead, it is transiently required for the differentiation of neural progenitors, but has to be inhibited for the further differentiation of neuronal precursors. Also, Wnt is a negative regulator for tanycyte gliogenesis (Fig. 4.2).

I also performed comparative analyses between the adult zebrafish and adult mouse hypothalamus. Although I showed the conservation of hypothalamic Wnt activity, the genetic tools we selected were uninformative with respect to adult neuronal differentiation, as discussed in Chapter 3. Using *Hes1*^{CreERT2} as the driver to perform loss-of-function (LOF) and gain-of-function (GOF) experiments in the Wnt signaling pathway, we failed to detect any newborn neurons from this lineage even after nine months of tracing. There are two possible explanations for this observation. The first possibility is that there are no or at best very few neurogenic events in the adult mammal hypothalamus during homeostasis. All evidence suggesting that there is ongoing neurogenesis in the adult mammalian hypothalamus comes from BrdU tracing experiments. It is possible that those BrdU labeled cells were actually subtypes of later

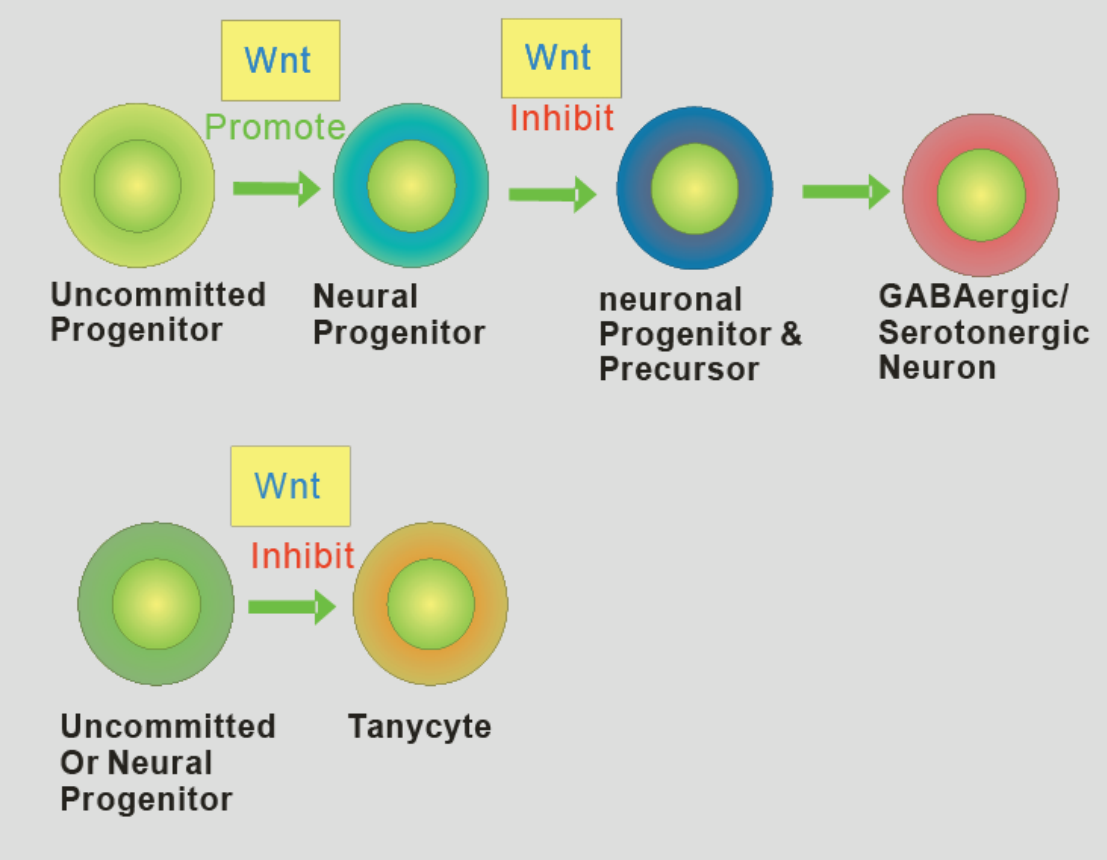

Figure 4.2 The role of the Wnt signaling pathway in post-embryonic neurogenesis and gliogenesis. Wnt is transiently required for neurogenesis and generally inhibits gliogenesis of tanycytes.

neuronal precursors; thus the so-called newborn neurons (based on BrdU uptake) do not actually arise from a progenitor population in the SVZ. In fact, there is no genetic evidence that a hypothalamic neuron is differentiated from a neural progenitor in adulthood. The second possibility is that there are some Hes1^{-} neural progenitors existing in the hypothalamic ventricular zone. If that is the case, several different Cre-drivers that are expressed in neural progenitors would be good candidates for further functional analysis: *Nestin*^{CreERT}, *Sox2* ^{CreERT2}, *Ascl1*^{CreERT}, and *GFAP*^{CreERT2}.[2-5] In particular, the hypothalamic specific regional driver *Nkx2.1*^{Cre} would also be an ideal candidate since *Nkx2.1* is the most specific gene expressed in the entire embryonic hypothalamus, as well as in the progenitor pool of the hypothalamus at later stages.[6, 7] However, a previous *Nkx2.1*^{Cre} mouse line was made from BAC transgenesis, and a *nkx2.1a:YFP* zebrafish line was a retroviral insertion, so the optimal tool to make for this analysis would be to isolate a hypothalamus-specific *Nkx2.1* enhancer.

Over the course of my graduate studies, I also investigated the zebrafish *lef1* mutant that was generated by a previous postdoc in our lab, Junji Lin, using Zinc finger nucleases (ZFNs). Given the important functional role of the canonical Wnt signaling pathway in early development, the phenotype of this mutant at early stages is surprisingly mild: the embryo is phenotypically normal, and the larva only has a disrupted fin shape at later stages. However, *tcf7* and *tcf4* are known to be functionally redundant to *lef1*, and the *tcf7* mutant itself has an even milder phenotype[8-10]. Robert Duncan is currently working on the phenotypes of the double mutant of *lef1* and *tcf7*, and

we expect to see a severe phenotype in brain neurogenesis.

The significance of the work and future directions

Although my work in this thesis has exhaustively described the role of Wnt signaling in the zebrafish hypothalamus, there are two further questions: 1) what circuitry and behaviors do the Wnt-responsive newborn neurons contribute to in the adult hypothalamus; 2) what are the transcriptional targets of canonical Wnt signaling pathway in the hypothalamus or for that matter, the entire CNS.

For the question 1, we know that the brain is not static in its organization, and the zebrafish hypothalamus may require constitutive neurogenesis to support this structural and functional plasticity throughout life. With depletion of newborn neurons, the adult hypothalamus gradually loses its morphology and function. It is hypothesized that under this condition, an animal's metabolism will no longer be able to maintain homeostasis in response to changes in the environment, and that resulting effects on the hypothalamus will cause changes in food and water intake regulation, sleep-wake cycle regulation, and other endocrine functions. GABAergic, dopaminergic, and serotonergic neural systems in the hypothalamus have been suggested to be involved in those activities, but a precise quantitative relationship has not been established. Once we are able to modify hypothalamic circuitry, we may be able to make life better for those who suffer from autonomic and endocrine dysfunction or obesity.

To address question 2, Junji Lin performed ChIP-Seq assays using the Lef1 antibody.

Interestingly, many candidate targets identified are associated with synaptic formation, such as *nrxn1a*, *nrxn2b,* and *sypa*. Further in-situ analyses have revealed that these genes are expressed in the hypothalamus, and some expression domains are adjacent to Wnt active regions. Given that a large proportion of the Wnt reporter cells and *lef1/tcf7* expressing cells overlap with the markers of later neuronal precursors and mature neurons, it is possible that one of the important roles of Wnt signaling is to regulate the synaptogenesis or initiate the transcription of synaptogenesis-associated components for neurons or neuronal precursors. However, this hypothesis is only supported by our ChIP data and has not yet been described by other groups.

Finally, the work described in this dissertation has significantly broadened our understanding of the hypothalamus as a model of neurogenesis not just at embryonic stages but also at the adult stage. My work also represents the first time that hypothalamus has been analyzed in two different species at different stages of development to address a single question: what is the role of Wnt signaling in neurogenesis? During the process of this project, I received a lot of advice from other researchers and their published articles, and have tried a variety of reagents and genetic tools to test our hypothesis. Some of the conclusions I have drawn, specifically the conclusion that gain and loss of Wnt signaling will inhibit the completion of neurogenesis, challenge existing models. However, I have been able to make a model of hypothalamic neurogenesis that is being corroborated in recent publications from other laboratories.

In the end, I have a feeling: biology is soft and fluid, and the modeling of biologic function does not suit the artificiality of our mathematics system; we may need to learn the nature of biological mathematics or design a new one: biomathematics.

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